Expression of ryanodine receptors in human embryonic kidney (HEK293) cells

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It has been shown previously that mobilization of caffeine-sensitive intracellular calcium (Ca^{2+}_i) stores increased the release of amyloid β-peptide (Aβ) from transfected human embryonic kidney cells (HEK293) [Querfurth, Jiang, Geiger and Selkoe (1997) J. Neurochem. **69**, 1580–1591]. The present study was to test the hypothesis that the caffeine/Aβ responses were due to interactions with specific subtypes of ryanodine receptors (RyR) using [^{3}H]ryanodine receptor binding, epifluorescence imaging of Ca^{2+}_i, immunocytofluorescence, immunoprecipitation and PCR techniques. [^{3}H]Ryanodine bound to a single class of high-affinity caffeine-sensitive sites ($K_d = 9.9 \pm 1.6$ nM, $B_{max} = 25 \pm 4$ fmol/mg of protein). RyRs were immuno-decorated in a punctate reticulo-linear pattern. Results from SDS/PAGE and reverse transcriptase-PCR demonstrated endogenous expression of type 1 (skeletal) and type 2 (cardiac) RyRs. HEK293 cell RyRs were functionally active, because (i) $[Ca^{2+}]_i$ increased 2.8-fold over baseline following applications of 5–15 mM caffeine, (ii) repetitive spiked increases in $[Ca^{2+}]_i$ were observed, and (iii) evidence for a use-dependent block was obtained. Some of these findings were extended to include HeLa and human fibroblast cell lines, suggesting a broader applicability to cells of epithelioid lineage. Implications for the processing of the β-amyloid precursor protein in Alzheimer's disease and for calcium channel research using transfected HEK293 cells are discussed.

INTRODUCTION

Recently we reported that ryanodine receptor (RyR) agents caffeine and ryanodine increased amyloid-β peptide (Aβ) production by a human epithelioid and embryonic kidney (HEK)293 cell line [1]. RyRs are a family of trans-membrane tetrameric proteins that serve as intracellular calcium (Ca^{2+}_i) release channels. In vertebrates, RyR1 predominates in skeletal muscle and Purkinje cells of the cerebellum, RyR2 is found in cardiac muscle and brain, and RyR3 is found in smooth muscle and the corpus striatum and limbic cortex of brain [2]. RyRs serve to amplify Ca^{2+}_i signals through a process termed calcium-induced calcium release (CICR) [3–5].

The plant alkaloid ryanodine alters channel gating and conductance at low concentrations ($< 10\ \mu$M) by activating the channel and stabilizing it in a subconductance state, whereas higher concentrations ($> 100\ \mu$M) lock the channel in a closed state [6,7]. The methylxanthine, caffeine, sensitizes RyRs to cytosolic Ca^{2+} and potentiates the maximal rate of Ca^{2+}_i release at optimal calcium concentrations [2]. In addition to its stimulatory and permissive effects on RyRs and CICR, caffeine has also been shown to inhibit Ca^{2+} release from inositol 1,4,5-trisphosphate (IP_3)-sensitive pools at concentrations that induce Ca^{2+} release from RyRs [8].

Calcium signalling in non-excitable cells has traditionally been ascribed to 'capacitative' calcium influx and agonist-induced release of calcium from intracellular stores bearing the ubiquitous IP_3 receptor [9,10]. Recently, RyR gene expression has been found in several non-excitable tissues and cells [11–13]. Single reports described transforming growth factor β-induced expression of a caffeine-insensitive type 3 RyR isoform in mink lung epithelial cells [14], and RyR binding in bovine corneal epithelial cells [15]. However, RyR expression does not imply production of functioning caffeine-sensitive calcium-release channels or effects on cellular metabolism. For one non-excitable cell type, epithelial cells, RyR activation could play a role in the regulation of cell differentiation and proliferation through calcium-requiring signalling pathways that do not require inositol lipid hydrolysis [16].

In this report the hypothesis, that specific subtypes of endogenous RyRs in human embryonic kidney (HEK)293 cells form functional Ca^{2+}-release channels, which interact with the Ca^{2+}_i mobilizer caffeine, was tested. It was important to confirm this hypothesis experimentally because recent results have shown that caffeine increased Aβ production and release independently of IP_3 and cAMP in HEK293 cells stably transfected with the β-amyloid precursor protein (βAPP) [1]. We have extended some of our findings to include HeLa and human fibroblasts and speculate that RyR expression may have physiological consequences across epithelial cell lines.

MATERIALS AND METHODS

Cultured human cells and rat tissue preparation

HEK293 (ATCC and Institutional sources), HeLa and normal human fibroblast cell lines (AGO 7095, 9309; Coriell Institute, Camden, NJ, U.S.A.) were grown in 80-cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal-calf serum. Plated cells were grown in a humidified 37 °C, 5% CO_2/95% air environment to a density of

Abbreviations used: HEK, human embryonic kidney; IP_3, inositol 1,4,5-trisphosphate; RyR, ryanodine receptor; CICR, calcium-induced calcium release; Ca^{2+}_i, intracellular calcium; Aβ, amyloid β-peptide; βAPP, β-amyloid precursor protein; DMEM, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody; DTT, dithiothreitol; AM, acetoxymethyl ester; sRyRs, solubilized ryanodine receptors; PDI, protein disulphide-isomerase; ER, endoplasmic reticulum; BAPTA, bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; RT, reverse transcriptase.

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approx. 10^5 cells/well. Membranes from rat brain cerebral cortex were prepared exactly as described in [17] and used at a protein concentration of approx. 3 mg/ml.

[^{3}H]Ryanodine binding

Separate binding assays ($n = 6$) were each conducted in duplicate in a final assay volume of 1 ml as described previously [17]. Specific binding was determined by subtracting binding with 15 nM [^{3}H]ryanodine plus 50 nM unlabelled ryanodine (non-specific binding) from binding with 15 nM [^{3}H]ryanodine (total binding). In labelled titration/equilibrium binding experiments, [^{3}H]ryanodine was used at concentrations of 1.4–51 nM.

Immunocytofluorescence

Monoclonal antibody (mAb) XA7B6, an IgM, was raised against rabbit skeletal-muscle triads and specifically recognizes junctional sarcoplasmic calcium-release-channel–ryanodine-receptor complexes [18]. Anti-protein disulphide-isomerase (PDI) (Affinity Bioreagents) is a mAb raised against the KDEL-motif-bearing endoplasmic reticulum (ER) marker PDI. An IgG1 antibody, mAb 34C (Affinity Bioreagents), against avian striated muscle specifically recognizes RyR1 and RyR2 isoforms in a number of mammalian species [19]. The dilutions used for immunocytochemistry were 1:20 for XA7B6 and 1:100 for PDI. Goat anti-mouse IgG conjugated with CY3 (indocarbocyanine; Jackson ImmunoResearch) at 1:250 dilution was used as a secondary antibody. HEK293 and human fibroblasts, grown to between 50 and 80 % confluence on poly-D-lysine-coated coverslips, were fixed in 4 % (w/v) paraformaldehyde/4 % (w/v) sucrose in PBS for 20 min at 21 °C, and permeabilized with 0.3 % (v/v) Triton X-100 for 5 min. Cells were blocked in 10 % (v/v) goat serum/PBS for 1 h at 21 °C. Coverslips were inverted on to 50 μl of primary antibody in a humidified chamber for 2–3 h at 21 °C or overnight at 4 °C. They were washed three times with PBS and secondary antibody was applied for 1 h at 21 °C; the coverslips were then mounted on to microscope slides in 4 % (w/v) *N*-propyl gallate/66 % (v/v) glycerol in PBS and photographed using an inverted fluorescence light microscope (Nikon Diaphot) at 100 × magnification. For CY3 assay, the excitation filter was 510–560 nm and the barrier filter (Rhodamine) was 590 nm. Frozen sections of fresh normal human skeletal muscle (10 μm) were fixed in acetone for 2 min at 4 °C, rinsed three times in PBS and immunostained as desribed above.

Immunoprecipitation and Western blotting

Microsomes were prepared and RyRs were solubilized as described previously [20]. Four large flasks (150 cm^2) containing HEK293 or HeLa cells were metabolically labelled overnight in 20 ml of medium containing 20 μCi/ml [^{35}S]methionine (NEN). Unlabelled or [^{35}S]methionine-labelled HEK293 and HeLa cells were lysed in 10 ml of buffer [10 mM imidazole/0.3 M sucrose/5 mM dithiothreitol (DTT)/230 μM PMSF/1 μM leupeptin, pH 7.4], the final protein concentration was adjusted to 5 μg/μl and the samples were stored in liquid N_2. The lysates were thawed quickly and microsomes were pelleted by centrifugation at 100000 g_{max} for 90 min. Microsomal fractions were homogenized in buffer containing 0.5 M KCl, 20 mM Tris/HCl, 5 mM DTT, 5 % CHAPS and 2.5 % L-phosphatidylcholine (pH 7.4), centrifuged at 100000 g_{max} for 30 min, and supernatants containing solubilized ryanodine receptors (sRyRs) were stored in liquid N_2 at a final protein concentration of 5.8 mg/ml (HEK293) or 2.4 mg/ml (HeLa). Crude microsomal membranes, prepared from chicken pectoral muscle were solubilized and the final protein concentration was adjusted to 1.4 mg/ml. Immunoprecipitation of sRyRs was performed essentially as described in [19,21]. mAb 34C was incubated at a 1:20 dilution overnight at 4 °C with goat anti-mouse IgG-linked agarose beads (750 μl original slurry; Sigma). Antibody-coupled beads were washed 3 times in PBS, resuspended in buffer and aliquots of 200 μl were incubated with approx. 8 ml of HeLa or HEK293, or 1.25 ml of chicken pectoral muscle sRyRs overnight at 4 °C. Pre-clearing the microsomes with 100 μl of agarose beads for 2 h reduced background interference. For SDS/PAGE, beads were washed in PBS containing 0.5 % (v/v) CHAPS and 0.25 % L-phosphatidylcholine and suspended in 100 μl of 2 × sample buffer containing 200 mM Tris/HCl (pH 6.8), 12 % (v/v) glycerol, 4 % (w/v) SDS and 14 μl of 1 M DTT. For Western blotting, 45 μl of chicken pectoral muscle sRyRs was mixed directly with DTT and sample buffer. Samples were concentrated to 50 μl (8 % SDS) before gradient (4–20 %) or 4 % acrylamide SDS/PAGE. Proteins were electrotransferred on to nitrocellulose at 35 V and 100 mA overnight in running buffer (25 mM Tris base/0.2 M glycine) containing 10 % (v/v) ethanol and 0.02 % (w/v) SDS and were revealed by either autoradiography or immunodetection with 34C (1:500) and horseradish-peroxidase-conjugated goat-anti mouse IgG (1:1000; Pierce) followed by enhanced chemiluminescence (Amersham).

Fluorescence imaging of Ca^{2+}_i

HEK293 cells on coated glass coverslips were loaded with 10 μM Fluo-3/AM (acetoxymethyl ester) (Molecular Probes) in serum-free DMEM at 37 °C, 5 % CO_2/95 % air for 45 min, washed twice in DMEM and analysed using an interactive laser cytometer (ACAS 570; Meridian Instruments, Okemos, MI, U.S.A.) [22]. Individual cells were imaged at 15 s intervals with an excitation wavelength of 488 nm and an emission wavelength of 530 ± 15 nm. The integrated signal from all pixels within each cell boundary was taken as the relative measure of Ca^{2+}_i for that cell. Measurements were made at 21 °C in 1.0 ml DMEM (3 mM Ca^{2+}) in which various concentrations of caffeine (0.15 M stock in DMEM, heated to 42 °C before use) or A23187 (5 mM stock in DMSO) were premixed. The cells were washed for 15 min with DMEM (and vehicle) between drug applications to bring signals back to baseline.

For quantitative measures of Ca^{2+}_i, HEK293 cells were loaded with 2 mM of the calcium-sensitive dye Fura-2/AM (Molecular Probes) for 40 min at 21 °C in Krebs buffer (111 mM NaCl/26.2 mM $NaHCO_3$/1.2 mM NaH_2PO_4/4.7 mM KCl/1.2 mM $MgCl_2$/1.8 mM $CaCl_2$/5 mM D-glucose/15 mM Hepes, pH 7.4). Ca^{2+}_i imaging was accomplished using a dual excitation (340/380 nm), single emission (510 nm) system (Empix Imaging Inc., Mississauga, ON, Canada). Images were acquired every 5 s by real time averaging of 16 frames for each wavelength. In a typical experiment, cells maintained at 37 °C were superfused with buffer containing the appropriate vehicle [ethanol 0.05 % (v/v); ryanodine or DMSO 0.2 % (v/v); bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA)] at the rate of 2 ml/min. Superfusion was stopped before application(s) of 100 μM ryanodine or 15 mM caffeine to the bath and was continued for 4 min between drug applications. $[Ca^{2+}]_i$ increases were calculated as the difference between baseline (an average of the last three recorded $[Ca^{2+}]_i$ before drug additions) and peak Ca^{2+}_i responses.

Isolation of total RNA and reverse transcriptase (RT)-PCR

Total RNA was isolated from HeLa and HEK293 cells using the total RNA isolation kit (S.N.A.P., Invitrogen). RNA (1 μg) was reverse transcribed using 100 ng/μl oligo(dT) primers and 200

units of Moloney murine leukaemia virus reverse transcriptase (RT) (Gibco/BRL) in the presence of 6.7 mM DTT and 3.33 mM of a dNTP mixture in a total volume of 60 μl. PCR was performed using primers specific for each of the three RyR isoforms, RyR1 (PR3 and PR4), RyR2 (PR1 and PR2) and RyR3 (PR5 and PR6) [13]. First-strand cDNA (2 μl) was added to 200 μM dNTP mixture, 2 mM $MgCl_2$, 1 μM of each primer and 2.5 units of Taq DNA polymerase (Gibco/BRL) in a final reaction volume of 100 μl. The PCR was performed with 30 amplification cycles: 45 s at 94 °C, 45 s at 56 °C, followed by 1 min 10 s at 72 °C. The final cycle was followed by a 10 min elongation step at 72 °C followed by cooling to −9 °C. Reaction products (20 μl) were examined on a 1% agarose (Gibco/BRL) 1 × 40 mM Tris/20 mM acetate/1 mM EDTA, pH 7.2 gel stained with ethidium bromide containing a 1 kb DNA ladder size marker (Gibco/BRL). Control reactions were performed by omitting RT in the cDNA synthesis step. The presence of mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to assess the quality of the cDNA preparations. RT-PCR was performed using the 20-mer sense primer, 5′-GGATGACCTTGCCCAC-AGCC-3′ and the 20-mer antisense primer, 5′-GCTGGGGCT-CACCTGAAGGG-3′ and the conditions described above [23] .

Statistics

Values for [^{3}H]ryanodine binding affinity (K_d) and density (B_{max}) were determined using nonlinear curve-fitting regression analyses (Prism, Graphpad). $[Ca^{2+}]_i$ was determined using the following equation:

$$[Ca^{2+}]_i = K_d \cdot (R - R_{min})/(R_{max} - R) \cdot \beta$$

where K_d stands for the apparent dissociation constant of Fura-2 for Ca^{2+}, R_{max} and R_{min} are ratio (340/380 nm) values in the presence of an excess amount and virtual absence of Ca^{2+} (Krebs, no added Ca^{2+}, 2 mM EGTA) respectively, and β refers to the ratio of the fluorescence intensity coefficient at 380 nm in the virtual absence of Ca^{2+}. Unless otherwise indicated, significance levels were determined by one-way analysis of variance (ANOVA) followed by the Tukey method for multiple comparisons.

RESULTS

[^{3}H]Ryanodine binding

[^{3}H]Ryanodine is a highly specific ligand that binds to the calcium-release channels of RyRs. [^{3}H]Ryanodine binding to HEK293 cells in buffer containing high salt (1.0 M KCl) and 100 μM $CaCl_2$ was specific to a single class of sites with apparent values for K_d of 9.9 ± 1.6 nM and B_{max} of 25 ± 4 fmol/mg of protein. Compared with binding under low salt conditions (200 mM KCl), values for the apparent B_{max} in high salt were 3-fold higher in HEK293 cells and 2-fold higher in rat brain (n = 11 assays, each in duplicate; results not shown). Under low salt conditions (200 mM KCl), binding was submaximal, as expected, and 20 mM caffeine significantly increased [^{3}H]ryanodine binding from basal levels of 7 ± 1 to 12 ± 2 fmoles/mg protein in HEK293 cells ($P < 0.001$) and from basal levels of 52 ± 7 to 71 ± 8 fmol/mg of protein in rat brain cortex ($P < 0.01$). At 9.9 nM (K_d), [^{3}H]ryanodine-specific binding, expressed as a percentage of total binding, was 7 ± 2% for HEK293 cells and 38 ± 3% for rat brain membranes assayed in parallel.

Immunofluorescence

The mAb XA7B6 produced a modest punctate staining pattern within the cytosol and cell processes as well as a finely 'peppered' decoration over central areas in 80–90% of HEK293 cells (Figure 1A). The punctate staining often appeared in short radiating linear strands (indicated with arrows). Antibody specificity was confirmed by the complete lack of fluorescent signal in the absence of primary antibody or when control mouse ascites fluid was used as primary antibody and the characteristic localization of RyRs at the junctional triad in human skeletal muscle (results not shown). The specificity of mAb XA7B6 has been extensively documented using [^{3}H]ryanodine binding to immunoprecipitates of skeletal-muscle triads [18] and Western-blot analysis [24]. The soluble ER marker PDI, robustly stained HEK293 cells with a 'wispy', non-punctate, cytoplasmic and peri-nuclear reticular fluorescence (Figure 1B). The XA7B6 pattern in HEK293 cells therefore could not be identified specifically with the ER; however, it was not inconsistent with the possibility of a finite number of membrane-bound RyRs clustered within the ER. Similar results were obtained in HEK293 cells using antibody 34C at a 10-fold greater dilution than used with XA7B6 (results not shown).

Immunoprecipitation and Western blotting

The receptor binding data indicated a level of RyR expression in HEK293 cells that was an order of magnitude less than that observed in rat brain. It was expected that HEK293 RyR1 expression would be several orders of magnitude less than in skeletal muscle (see [12]). Indeed, using standard Western-blot analysis, endogenous RyR expression could not be demonstrated in HEK293 microsomal fractions (results not shown), whereas it was robust in avian skeletal muscle (see Figure 3B, lane 2). In fact, Western blots using a whole cell extract or microsomal fraction demonstrated no protein staining with 34C, whereas non-specific staining of mid-range molecular-mass proteins (60–200 kDa) was observed when developed with polyclonal anti-RyR (results not shown). However, low levels of endogenous RyR expression were identified in labelled HEK293 cells when microsomes were first immunoprecipitated with mAb 34C before SDS/PAGE and autoradiography (Figure 1C, lane 3). Detection was also possible in immunoprecipitation/immunoblots (Figure 1C, lane 7). The expanded upper region of the 4%-gel autoradiogram revealed two noticeable but weak, high-molecular-mass signals. These signals co-migrated with a similar broad band in a Western blot from the same gel of immunoprecipitated control avian skeletal-muscle microsomes (Figure 1C, lane 6). The narrow lower band(s) observed in lanes 3 and 7 could represent a degradation product, co-precipitant, artifact or the RyR2 isoform, which migrates ahead of the RyR1 isoform [12]. The specificity of mAb 34C has been extensively demonstrated [19,21]. Furthermore, mouse ascites-fluid control did not recognize RyRs in the immunoprecipitations (Figure 1C, lanes 2, 5 and 8), neither did mAbs against irrelevant Golgi membrane proteins (results not shown). 34C mAb was used in these experiments, rather than XA7, because the immunoprecipitation technique for ^{35}S-labelled, cultured cell microsomes had been published previously [19,20]. Therefore RyRs appeared to be at least an order of magnitude more abundant in avian skeletal muscle than in HEK293 cells, since 20-fold more microsomal protein was immunoprecipitated in HEK293 cells than in skeletal muscle in these experiments. However, the sensitivity of 34C mAb for human RyR isoforms is presently not known.

Ca^{2+}_i concentrations

Initially, we established maximal semi-quantitative $[Ca^{2+}]_i$ responses to various caffeine doses in HEK293 cells. Caffeine-induced increases in $[Ca^{2+}]_i$ were maximal in the 5–15 mM

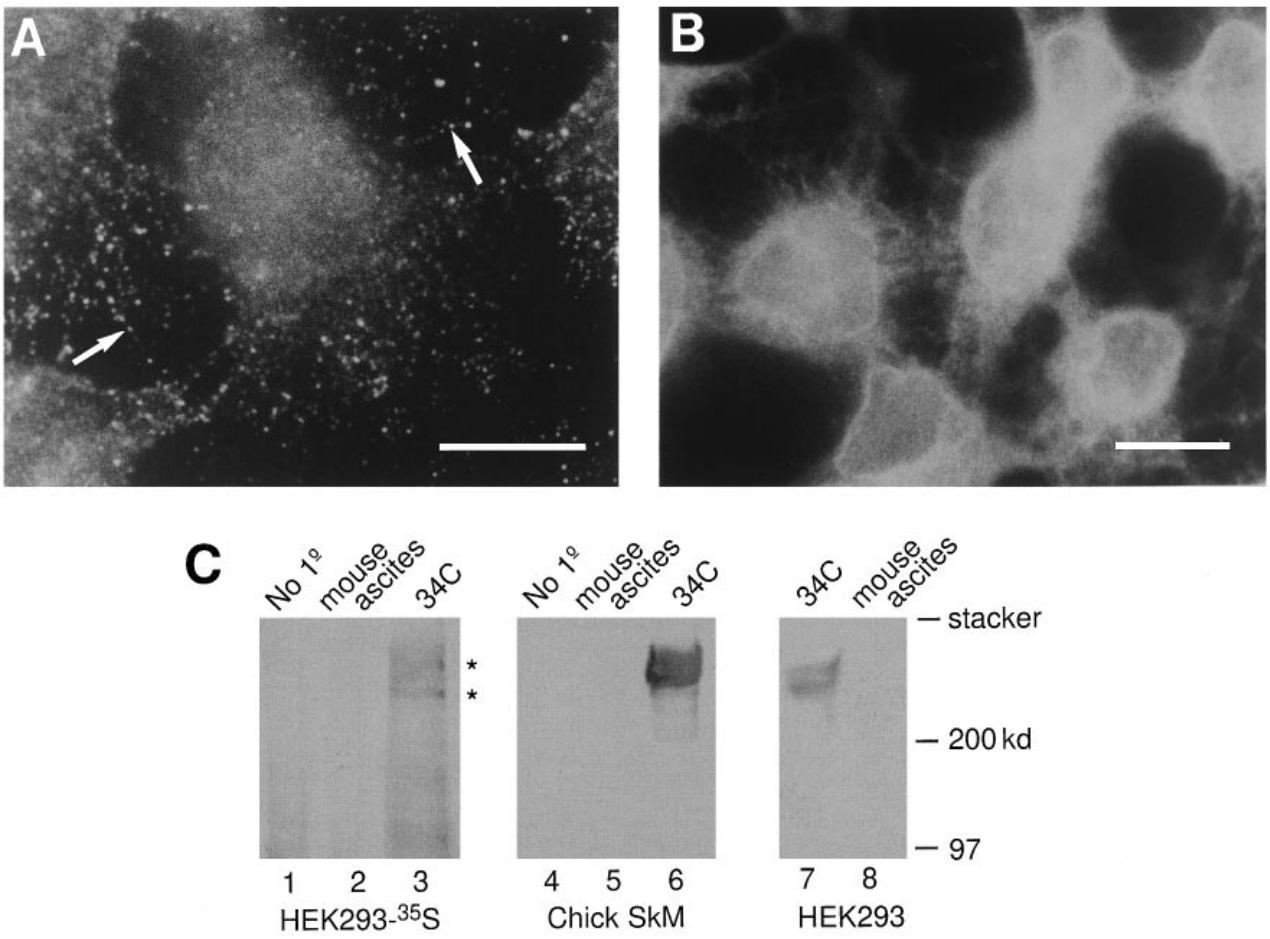

Figure 1 RyR decoration by immunocytofluorescence (A and B), and immunoprecipitation of RyRs in microsomal fractions followed by SDS/PAGE (C) in HEK293 cells

(**A**) HEK293 cells labelled with XA7B6 (1:20 dilution). The punctate XA7B6 decoration appeared linear-radial (arrow). (**B**) A fine reticular pattern was obtained using anti-PDI mAb. The same results were obtained using mAb 34C (results not shown). The bars at the bottom right of each panel represent 10 μm. (**C**) Lanes 1, 2 and 3, autoradiogram of immunoprecipitation of RyRs, using mAb 34C, from [^{35}S]methionine-labelled HEK293 (HEK293-^{35}S) microsomes (48 mg); lanes 4, 5 and 6, immunoblot of unlabelled chicken skeletal muscle (Chick SkM) microsomes (2 mg); and lanes 7 and 8, immunoblot of unlabelled HEK293 microsomes. Lanes 1 and 4, control immunoprecipitation with no primary mAb (No 1°); lanes 2, 5 and 8, control immunoprecipitation using mouse ascites fluid; lanes 3, 6 and 7, immunoprecipitation using mAb 34C. Samples were separated by PAGE (4% polyacrylamide gels) before autoradiography for 17 h at −80 °C or were analysed by Western blotting followed by detection by enhanced chemiluminescence. The high-molecular-mass doublets in lane 3 (*) and lane 7 co-migrated with the broad signal seen in lane 6. The positions of molecular-mass markers (kd = kDa) are shown on the right.

caffeine range. At 10 mM caffeine, the increase in $[Ca^{2+}]_i$ was approx. 2.8-fold in HEK293 cells ($n = 4$ cells). To demonstrate further the specificity of caffeine-induced $[Ca^{2+}]_i$ responses, non-saturating dose-dependent increases in $[Ca^{2+}]_i$ were observed with the ionophore A23187 (Figure 2A, inset).

In order to determine the effects of repeated caffeine applications on $[Ca^{2+}]_i$, maximum doses of caffeine (15 mM) were applied twice to HEK293 cells ($n = 23$ of 99 cells). The first application of caffeine resulted in a sharp increase in $[Ca^{2+}]_I$, which averaged 484 ± 176 nM, followed typically by a prolonged rise in $[Ca^{2+}]_i$ above baseline. A second application of 15 mM caffeine produced a smaller increase in $[Ca^{2+}]_I$, which averaged 275 ± 85 nM (Table 1). The differences between the first and second responses were not statistically significant. In the absence of external calcium, the maximal amplitude of $[Ca^{2+}]_i$ increase was not significantly different from the spike observed in calcium-containing buffer, which establishes release from intracellular stores. However, the prolonged phase of increase, which immediately followed the spike in calcium-containing buffer, was absent (Figure 2A and Table 1). When the rapid $Ca^{2+}{}_i$ chelator BAPTA/AM (20 μM) was loaded before stimulation, both calcium responses were abolished, as expected (Table 1). Repetitive applications of caffeine in calcium-free buffer resulted in a significant progressive attenuation of responses (Table 1).

A variety of $Ca^{2+}{}_i$ responses occurred following caffeine additions in HEK293 cells that were pretreated with ryanodine. These responses were classified according to the number of spikes in $[Ca^{2+}]_i$ that occurred following the first addition of caffeine. Single, double and multiple responses were identified. Double and multiple $[Ca^{2+}]_i$ spikes following caffeine were not observed unless the cells were first pretreated with ryanodine. The presence of 100 μM ryanodine produced increases in peak $[Ca^{2+}]_i$ of 318 ± 24 nM. After $[Ca^{2+}]_i$ stabilized, superfusion with 15 mM caffeine resulted in single spike increases in $[Ca^{2+}]_i$ of 516 ± 170 nM in 14 of 111 cells tested. Responses to second additions of 15 mM caffeine were significantly smaller ($P < 0.05$) (Figure 2B and Table 2). In all ryanodine-pretreated cells, $[Ca^{2+}]_i$ responses to the second application of caffeine were significantly

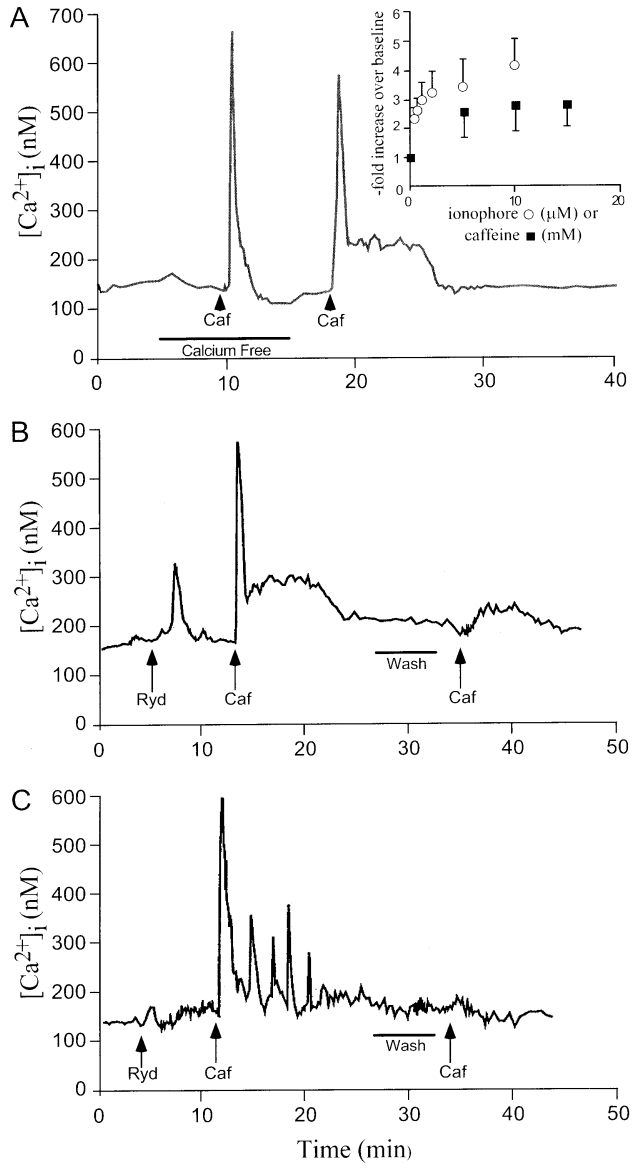

Figure 2 Fluo-3/AM fluorescence traces of $[Ca^{2+}]_i$ in HEK293 cells

(**A**) Fluo-3/AM fluoresence trace of $[Ca^{2+}]_i$ in HEK293 cells showing similar peak responses following 15 mM caffeine (Caf) treatment in the absence and presence of extracellular Ca^{2+}. A plateau was apparent following peak increases in Ca^{2+}-containing but not in Ca^{2+}-free Krebs buffer. Maximal doses of caffeine were used in all experiments, producing a 2.8-fold increase in $[Ca^{2+}]_i$ over baseline (inset). (**B**) Representative trace from 1/14 HEK293 cells pretreated with ryanodine (Ryd) showing a single spike response to the first application of caffeine (Caf). (**C**) Representative trace obtained from 1/20 cells pretreated with ryanodine (Ryd) showing three or more regenerative spikes in $[Ca^{2+}]_i$ following the first application of caffeine (Caf).

smaller ($P < 0.05$) than those produced by a second application of caffeine in cells not pretreated with ryanodine (Table 2).

In a separate cohort of cells, where two distinct increases in $[Ca^{2+}]_i$ were observed following caffeine (45/111 cells), ryanodine-induced increases in $[Ca^{2+}]_i$ were 143 ± 2 nM, which was significantly less ($P < 0.01$) than peak $[Ca^{2+}]_i$ increases following ryanodine applications in single spike cells (Table 2).

In 29 of 111 cells tested, three or more $[Ca^{2+}]_i$ spikes were observed following the first addition of caffeine. Ryanodine application alone resulted in $[Ca^{2+}]_i$ increases that averaged 163 ± 26 nM (results not shown). Following the application of

Table 1 Caffeine increased $[Ca^{2+}]_i$ in HEK293 cells

Caffeine (15 mM) applications, in either calcium-containing buffer or calcium-free buffer where indicated, were interspersed by four-min wash periods. Values represent increases over baseline levels of $[Ca^{2+}]_i \pm 1$ S.D., n = number of responsive cells exhibiting single spikes/total cells. The vehicle was 0.2% (v/v) DMSO.

Treatment	Increase in $[Ca^{2+}]_i$ (nM)
Caffeine application I	484 ± 176
Caffeine application II ($n = 23/99$)	275 ± 85
Caffeine application I (calcium free)	674 ± 78
Caffeine application II ($n = 50/160$)	565 ± 69
Caffeine (BAPTA/AM pretreatment) ($n = 112$)	3 ± 0.7
Caffeine (vehicle pretreatment) ($n = 37/148$)	416 ± 54
Caffeine application I (calcium free)	504 ± 68
Caffeine application II (calcium free)	21 ± 4
Caffeine application III ($n = 73/220$)	471 ± 63

Table 2 Ryanodine preconditioning inhibited subsequent caffeine-induced increases in $[Ca^{2+}]_i$ in HEK293 cells

The increase in $[Ca^{2+}]_i$ in HEK293 cells after application of ryanodine (100 μm) and caffeine (15 mM) are summarized as changes over basal levels ($\pm$S.D.) for cells exhibiting single and double spikes (concentrations in cells that exhibited more than two calcium peaks are not shown here; e.g. see Figure 7C). n = Number of responsive cells/total cells.

Treatment	Increase in $[Ca^{2+}]_i$ (nM)
Single $[Ca^{2+}]_i$ spikes	
Ryanodine application I	318 ± 24
Caffeine application II	516 ± 170
Caffeine application III ($n = 14/111$)	72 ± 14
Double $[Ca^{2+}]_i$ spikes	
Ryanodine	143 ± 3
Caffeine application I, spike 1	293 ± 76
Caffeine application I, spike 2	180 ± 76
Caffeine application II ($n = 45/111$)	76 ± 10

15 mM caffeine, up to six distinct $[Ca^{2+}]_i$ spikes of oscillating amplitudes were noted (Figure 2C). The amplitude of the first two spikes was less when compared with single-spike amplitudes in the absence or presence of ryanodine pretreatment ($P < 0.01$ in both cases).

PCR of RyR mRNA

According to Tunwell and Lai [13], the isoform-specific primer pairs are predicted to amplify a 903-bp region of RyR1 (PR3 and PR4), a 906-bp region of RyR2 (PR1 and PR2) and a 765-bp region of RyR3 (PR5 and PR6). In HEK293 cells approx. 900-bp products were produced with both the RyR1 and RyR2 primers, but no PCR product was amplified using RyR3 primers (Figure 3A). Control reactions, in which RT was omitted from the cDNA synthesis step, did not result in any amplification. Further evidence of specificity was obtained when positive control reactions for all three RyR isoforms were carried out using human fetal neurons for RyR1, rabbit heart for RyR2 and rabbit brain for RyR3. The masses of the PCR products from these control reactions corresponded exactly with the masses of the PCR products of the HEK293 cells (results not shown). Secondly, when HeLa cell mRNA was reverse transcribed and amplified, an approx. 900-bp product was detected with the RyR2 primers alone; no PCR products were observed in reactions where either

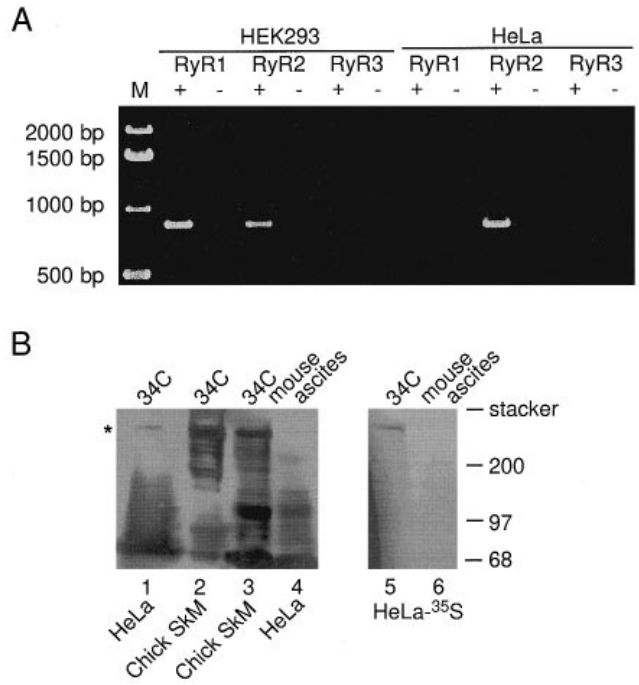

Figure 3 RT-PCR detection of RyRs in HEK293 and HeLa cells and immunoprecipitation and Western-blot analysis of RyRs in HeLa cells

(**A**) RT-PCR detection of RyRs in HEK293 and HeLa cells. DNA fragments of the appropriate mass were detected for RyR1 and RyR2 in HEK293 cDNA and for RyR2 only in mRNA from HeLa cells. +, RT present, or −, RT absent from cDNA synthesis step. M, bp DNA ladder. These results are representative of experiments using two different RNA preparations. (**B**) Immunoprecipitation and autoradiogram of Western blot of RyRs in HeLa cells. Lanes 1 and 4, unlabelled and lanes 5 and 6, ^{35}S-labelled HeLa cell microsomes (18 mg); lane 3, chicken skeletal muscle (Chick SkM) microsomes (2 mg) were immunoprecipitated using mAb 34C/goat anti-mouse-Sepharose, subjected to gradient (4–20%) PAGE and either immunoblotted using mAb 34C and rabbit anti-mouse-conjugated horse-radish peroxidase followed by detection with enhanced chemiluminescence (lanes 1, 3 and 4) or autoradiographed (lanes 5 and 6). Untreated chicken skeletal-muscle (Chick SkM) microsomal RyR (63 μg) (lane 2). Negative control immunoblot of HeLa microsomes using mouse ascites fluid as primary antibody (lane 4). A single, high molecular-mass band in lanes 1 (★) and 5 corresponded to the positive control bands in lanes 2 and 3.

the RyR1- or RyR3-specific primers were used (Figure 3A), which is in agreement with results obtained using a different set of primers [11].

RyR expression in other epithelioid cell lines

Western blots of solubilized RyRs immunoprecipitated from HeLa cell microsomal fractions revealed, on blots exposed for 15 min, a single, faint, high-molecular-mass band of approx. 350 kDa (upper-most band in lane 1, Figure 3B) that co-migrated with chicken skeletal-muscle microsomal fractions immunoprecipitated with 34C (Figure 3B, lane 3) (these were best distinguished on blots exposed 30 s; results not shown). A similar result was obtained when radioactively labelled immunoprecipitations were autoradiographed (Figure 3B, lane 5). The expression of RyRs by immunocytofluorescence and fluorescence calcium imaging in human fibroblasts was also determined. mAb XA7B6 (or 34C) produced radiating, reticulo-linear, punctate staining in the cytoplasm and perinuclear regions of approx. 1/3 of cells (results not shown).

In contrast with HEK293 cells, caffeine had no effect on $[Ca^{2+}]_i$, and ryanodine (100 μM) produced oscillatory $[Ca^{2+}]_i$ spiking in some fibroblast cells (results not shown). At ryanodine concentrations (10 μM) which had no intrinsic effect, modulation of calcium release by repeated applications of 1 mM ATP occurred. Approx. 75% of cells tested responded to application of ATP alone with an initial large release of Ca^{2+}_i (430 ± 70 nM), which subsequently stabilized at lower peak release levels (250 ± 50 nM) with repeated applications of ATP. When this ATP-responsive, sustainable calcium-release pool was tested for ryanodine sensitivity in another cohort of ATP-responsive cells, 60–70% of the cells displayed a significant progressive decrease in Ca^{2+}_i release with repeated ATP applications ($P < 0.001$). Similar data were reported for caffeine-insensitive HeLa cells, implicating a quantitatively low but functional level of RyR expression [11].

DISCUSSION

Functional RyRs are expressed in non-stimulated HEK293 cells. This conclusion is supported by the demonstration of: (i) high affinity binding sites for [^{3}H]ryanodine that are caffeine-sensitive, (ii) increased levels of Ca^{2+}_i following exposure to ryanodine or caffeine, (iii) detectable RyRs by immunocytofluorescence, (iv) a high-molecular-mass immunoprecipitable species that co-migrated with solubilized RyRs from skeletal-muscle microsomes and (v) expression of RyR1 and RyR2 message by RT-PCR. Our results support the hypothesis that caffeine accelerates Aβ production from transfected HEK293 cells by specific interaction with endogenous RyRs and stimulation of Ca^{2+}_i levels.

In HEK293 cells, we found that [^{3}H]ryanodine bound to a single class of receptors with an affinity constant comparable in magnitude (i.e. low nanomolar range) with that found in human brain membrane preparations [25]. Maximal [^{3}H]ryanodine binding was 4-fold less in HEK293 cells than that found in human cortical membranes [25] and 7-fold less than in the rat brain preparation data reported in the present work.

Endogenous RyR expression was detected in permeabilized HEK293 cells using mAb XA7B6. Subcellular localization appeared to speckle the cytoplasm in a pattern similar to that described for cultured developing rat myotubes [26] and rat ventricular cardiomyocytes [27]. We were not able to ascribe the expression of RyRs specifically to the ER in epithelial cells, since XA7B6 staining did not precisely co-localize with that of the soluble ER marker PDI. It is possible that the observed discontinuous pattern may correspond with localization of RyRs to subcellular compartments other than the ER, such as subspecialized ER cisternal vacuoles and calciosomes [28]. The relative difference in the RyR detection observed between the immunocytofluorescence results (Figure 1A) and the immunoprecipitations using 34C, where the signal was weaker (Figure 1C), is not easily explained. Because small amounts of very-high-molecular-mass proteins (> 500 kDa) cannot be transferred on to nitrocellulose efficiently, further quantification was not attempted. A direct comparison between mAbs XA7B6 and 34C was made by immunocytofluorescence, with very similar results. Immunoprecipitations were performed with the IgG mAb 34C, because of previously published technical details [19,21].

The RyRs detected in HEK293 cells had an apparent molecular mass of about 350 kDa, which was similar to earlier estimates of RyR size based on the results of SDS/PAGE [18]; however, revised estimates now place the mass closer to 595 kDa [2]. We did not attempt to identify the particular protein isoform(s) of RYRs expressed in HEK293 cells. It has been shown that 34C cross-reacts with the RyR1 and RyR2 isoforms [19,21], whereas XA7B6 does not appear to cross-react with the RyR2 isoform [24]. Therefore at least the RyR1 isoform is decorated in HEK293

cells and possibly RyR2 as well, a view supported by the immunoprecipitated doublet referred to in Figure 1. Since RyRs isolated from HEK293 cells and rat brain displayed caffeine sensitivity (a property not displayed by RyR3 isoforms [14]), it seems unlikely that RyR3 is present.

The predictions above, based on immunological data, were confirmed by RT-PCR, which revealed expression of RyR1 and RyR2, but not of RyR3 mRNA. Our results for HEK293 cells differ somewhat from the observations of Tunwell and Lai [13] who, using cDNA from rabbit kidney cortex and the rabbit kidney epithelial cell line LLC-RK1, detected only RyR2. This difference may be due to the fact that embryonic cells are less differentiated than the adult cells and possess both RyR1 and RyR2, but expression of RyR1 is lost with further differentiation. This hypothesis is supported by the results of Bennett et al. [11] using PC12 cells.

Caffeine has the pharmacological properties of an adenosine-receptor antagonist and phosphodiesterase inhibitor, with consequent elevation of cAMP. cAMP may regulate Ca^{2+}_i, although this is more usually by influx mechanisms such as non-specific cation and voltage-dependent calcium channels [29]. Therefore this mechanism cannot be responsible for the external calcium-independent spike phase seen in HEK293 cells, which ostensibly do not possess voltage-dependent calcium channels [30]. The caffeine effect that we report in HEK293 cells is most likely because of the well-known modulatory-ligand effect of activating Ca^{2+} release from RyR-regulated pools of Ca^{2+}_i.

In HEK293 cells, the Ca^{2+}_i response produced by a single application of caffeine consisted of an initial spike followed by a lesser sustained rise in $[Ca^{2+}]_i$. When the same saturating dose of caffeine was applied several times, the amplitude of each successive $[Ca^{2+}]_i$ spike modestly decreased, which was suggestive of either store depletion or receptor desensitization. In the absence of external calcium, we demonstrated the same Ca^{2+}_i transient, also consistent with RyR activation, but the delayed rise was abolished. Similar response patterns have been shown, using a variety of Ca^{2+}_i-mobilizing agents, where the spike was due to the release of Ca^{2+} from intracellular pools and the plateau phase was due to influx of extracellular Ca^{2+} [31]. Pretreatment of cells with the permeant Ca^{2+}_i-chelator BAPTA/AM abolished not only the spike but also the delayed rise, consistent with the 'capacitative entry' notion that influx is triggered after internal release.

We were able to demonstrate use-dependent RyR blockade, a significant reduction in the ability of repeated caffeine applications to elicit a Ca^{2+}_i response in cells pretreated with a high concentration of ryanodine. In addition to the single $[Ca^{2+}]_i$ spike commonly seen in response to caffeine, double and multiple spikes were observed when the cells were first exposed to ryanodine. Cells that displayed single $[Ca^{2+}]_i$ spikes were generally more sensitive to ryanodine pretreatment and produced higher initial caffeine-induced increases in $[Ca^{2+}]_i$, whereas the more populous regenerative spiking cells were less sensitive to ryanodine and showed smaller initial caffeine responses; the reason(s) for these differences is not known. Although CICR was not demonstrated here, it is not unreasonable to suggest from the results obtained that this mechanism for calcium release was present in HEK293 cells. It should be noted that regenerative calcium signals in non-excitable (including epithelial) cells can also be fulfilled by IP_3 receptors and their ligands [32]. Furthermore, activation of either the RyR or the IP_3 receptor can affect the other [5], and both receptors may utilize either the same [33] or segregated calcium stores [27,28].

Our demonstration of RyR expression in HEK293 cells obtained from two separate sources may be relevant to recent studies where this cell line was used to transfect RyR and other non-voltage-gated calcium channel proteins [34]. Sufficiently sensitive immunoprecipitation, PCR and quantitative individual cell fluorescence-imaging techniques, such as those used in the present work, may be required, depending on experimental circumstances, before it may be concluded that cells do not express RyRs.

Evidence for widespread expression of RyRs among other human cell lines of epithelial lineage was also obtained and included the novel demonstration of RyR detection by immunocytofluorescence and immunoprecipitation in fibroblasts and HeLa cells, a ryanodine-sensitive pool of Ca^{2+}_i in fibroblasts and RyR2 mRNA expression in HeLa cells using RT-PCR. Reports where other techniques have been used [11,12], and the data presented in the present work, have established the presence of RyR-regulated pools of Ca^{2+}_i in human epithelioid cells, raising the possibility that endogenous ligands such as cADP-ribose may participate in the regulation of $[Ca^{2+}]_i$ [35]. RyR activation could have a physiological role in the regulation of epithelial cell growth and differentiation in response to various mitogens, such as the epidermal growth factor receptor [36,37].

We have reported that caffeine and ryanodine stimulation of HEK293 cells transfected with the human βAPP gene resulted in an increased release of Aβ into the medium [1]. In the present work we present evidence to support specific activation of expressed and functional RyRs as the physiological basis for this observation. This may be the first example of an alteration in the metabolic processing of a cell surface receptor-like molecule (βAPP), which has been implicated in cell–cell adhesion and cell process extension [38], by RyR activation.

We are grateful to Dr. Kevin Campbell for supplying mAb XA7B6. We thank Dr. John Sutko for providing chicken skeletal-muscle microsomes and for helpful discussions. Donna-Marie Mironchuck helped with much of the photography and preparation of the Figures. This work was supported by grants from the National Institute of Neurological Disorders and Stroke (K08NS01737) to H.W.Q., from the National Heart, Lung and Blood Institute (R01HL32854 and P60HL15157) to D.E.G., and from the Alzheimer Society of Canada and Medical Research Council of Canada to J.D.G. N.J.H. was supported by a Studentship from the Alzheimer Society of Canada and J.D.G. was supported by an Medical Research Council of Canada Scientist award.

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Received 23 February 1998/7 May 1998; accepted 13 May 1998