HEK-293 cells possess a carbachol- and thapsigargin-sensitive intracellular Ca^{2+} store that is responsive to stop-flow medium changes and insensitive to caffeine and ryanodine

Jiefei TONG*, Guo Guang DU*, S. R. Wayne CHEN† and David H. MACLENNAN*[1]

*Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada M5G 1L6, and †The Cardiovascular Research Group, Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta, Canada T2N 4N1

Because HEK-293 cells are widely used for the functional expression of channels, exchangers and transporters involved in Ca^{2+} homoeostasis, the properties of intracellular Ca^{2+} stores and the methods used for measuring intracellular Ca^{2+} release in HEK-293 cells were evaluated. Ca^{2+} imaging was used to show caffeine-, carbachol- and thapsigargin-induced Ca^{2+} release in HEK-293 cells transfected with ryanodine receptor (RyR) cDNA, but only carbachol- and thapsigargin-induced Ca^{2+} release in untransfected HEK-293 cells. Intracellular Ca^{2+} release in untransfected HEK-293 cells was also observed if medium changes were performed by aspirating and replacing fresh medium (stop-flow), but not if medium changes were performed by a continuous over-flow procedure. Stop-flow medium-change-induced Ca^{2+} release in HEK-293 cells was independent of caffeine and ryanodine, demonstrating that it did not occur through RyR channels. Consistent with these observations was the observation that the level of expression of endogenous RyR proteins was below the limits of detection by Western blotting or [^{3}H]ryanodine binding. Thus the level of endogenous expression of RyR is so low in HEK-293 cells as to provide a negligible background in relation to functional analysis of recombinant RyR molecules. These results are inconsistent with those of Querfurth et al. [Querfurth, Haughey, Greenway, Yacono, Golan and Geiger (1998) Biochem. J. **334**, 79–86], who reported higher levels of endogenous RyR expression in untransfected HEK-293 cells.

Key words: Ca^{2+} photometry and imaging, Ca^{2+} release channels, ryanodine receptors.

INTRODUCTION

HEK-293 cells have become the mammalian expression system of choice for studying a variety of Ca^{2+} regulatory proteins. All three ryanodine receptor (RyR) isoforms have been expressed in HEK-293 cells and functional studies of mutant RyRs expressed in HEK-293 cells have generated important information on their structure, function and regulation [1–8]. However, the use of HEK-293 cells for the study of exogenous RyR proteins was recently questioned by Querfurth et al. [9], who reported that endogenous RyR protein is expressed in untransfected HEK-293 cells and that untransfected HEK-293 cells release Ca^{2+} in response to caffeine, an RyR-specific agonist. In contrast, we and others have used both Ca^{2+} photometry and Ca^{2+} imaging to show that Ca^{2+} is released in response to the addition of caffeine in HEK-293 cells transfected with RyR cDNA species but not in untransfected cells [3,5,6,8].

To achieve instant high concentrations of caffeine, Querfurth et al. [9] used a stop-flow protocol for caffeine application. Because it was not clear whether different protocols for caffeine application could influence Ca^{2+} release responses, thereby accounting for the different responses, we tested the effects of various modulators of Ca^{2+} release and of different protocols for medium changes on intracellular Ca^{2+} release in transfected and non-transfected HEK-293 cells. We present results showing that HEK-293 cells do not possess caffeine- and ryanodine-sensitive intracellular Ca^{2+} stores but that Ca^{2+} release can be induced in either transfected or untransfected HEK-293 cells by the stop-flow method of medium change used by Querfurth et al. [9]. These results, together with confirmatory functional and binding results, lead us to conclude that the level of endogenous expression of RyR is so low in HEK-293 cells as to provide a negligible background in relation to functional analysis of recombinant RyR molecules.

MATERIALS AND METHODS

Cell culture and DNA transfection

HEK-293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal serum at 37 °C under air/CO_2 (19:1). DNA transfection was performed by the calcium phosphate precipitation method [10]. Control cells were treated in the same way but with no DNA or with expression vector DNA [3]. The HEK-293 cells tested in this study were from an isolate used in our previous studies [1,3,5,6,8] and from a second isolate kindly provided by Dr Norman Haughey (University of Manitoba, Winnipeg, Manitoba, Canada). The latter was the same isolate as that used by Querfurth et al. [9]. The two isolates were compared in Ca^{2+} release measurements in response to agonists and to medium changes, and in measurements of [^{3}H]ryanodine binding.

Immunoblotting

Immunoblotting was performed on total proteins collected from one 60 mm plate of whole cell extracts [3]. The blots were incubated first with monoclonal antibody 34C at a dilution of 1:500 and then with horseradish-peroxidase-conjugated anti-mouse secondary IgG. Finally, the blots were incubated with the

Abbreviations used: RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase.

[1] To whom correspondence should be addressed (e-mail david.maclennan@utoronto.ca).

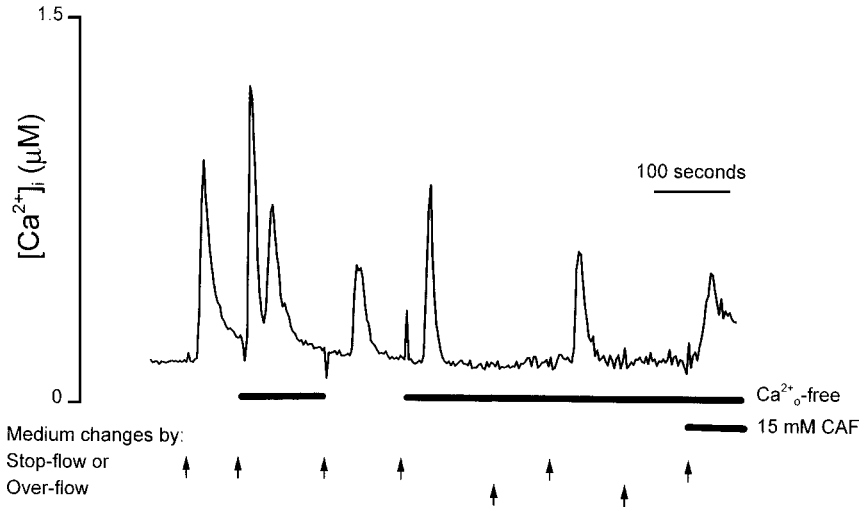

Figure 1 Stop-flow medium changes cause Ca^{2+} release in untransfected HEK-293 cells

Ca^{2+} imaging of an untransfected HEK-293 cell loaded with fura-2 was used to measure intracellular Ca^{2+} release. Medium changes were performed by either stop-flow (arrows in second row from bottom) or over-flow (arrows in bottom row) in the presence or absence (horizontal bars indicating Ca^{2+}-free medium) of Ca^{2+} in the medium. Ca^{2+} release was induced repetitively in the same cell when the medium was changed by stop-flow, in the presence and absence of medium Ca^{2+} and in the presence [horizontal bar indicating 15 mM caffeine (CAF)] and absence of 15 mM caffeine, but not by overflow, in a single fura-2-loaded, untransfected cell (representative of results from more than 50 of 150 cells examined). $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

SuperSignal ultra chemiluminescent substrate (Pierce) for 5 min and then exposed to a BioMax film (Kodak) [3].

Fluorescence measurements

A Photon Technologies microfluorimetry system was used to measure the effect of caffeine on Ca^{2+} release in HEK-293 cells [3,8]. In brief, Ca^{2+} photometry was performed on intact cells approx. 48 h after transfection. The cells were loaded with 4 μM fura-2 acetoxymethyl ester/0.02 % pluronic F-127 for 45 min at pH 7.4 and room temperature in a physiological medium. The glass coverslips were placed in a holder on the stage of an inverted Diaphot microscope (Zeiss). The samples were excited alternately at 340 and 380 nm with a dual monochromator. The emitted fluorescence was fed into a charge-coupled device camera (Photon Technologies SenSys-KAF 1400). Acquired digital images (400 ms per frame) were analysed with Image Master 2.0 software (Photon Technologies). The 340-to-380 nm ratios from single cells were converted into Ca^{2+} concentrations by the method of Grynkiewicz et al. [11]:

$$[Ca^{2+}] = K_d[(R-R_{min})/(R_{max}-R)](S_{f2}/S_{b2})$$

The R_{max} of 16 was obtained by using 10 μM ionomycin. R_{min} and the proportionality constant (S_{f2}/S_{b2}) were determined as 0.4 and 15.2 respectively. A value of 224 nM was used for the apparent K_d of Ca^{2+} binding to fura-2 [11].

Medium changes

Medium changes were performed either by the over-flow method described by Tong et al. [3] or by the stop-flow method of Querfurth et al. [9]. In the over-flow method, bathing solution containing the desired concentration of drug was added on one side of the stage and aspirated on the opposite side, so that cells were always covered by several millimetres of bathing solution. In the stop-flow method, which permits an instant change in the composition of the bathing solution, superfusion was stopped before application of an altered bathing solution, so that the cells were temporarily uncovered. Under these conditions of fluid replacement, however, the attached cells could be seen by microscopy or by imaging to be distorted.

[^{3}H]Ryanodine binding assay

[^{3}H]Ryanodine binding assays were performed as described previously [6]. In brief, supernatants from CHAPS-solubilized cells or from microsomes were incubated with 100 μM Ca^{2+} for 2 h at 37 °C in 0.25 ml of a solution containing 0.31–40 nM [^{3}H]ryanodine. The amount of [^{3}H]ryanodine bound was determined by filtration through Whatman GF/B membranes. Non-specific binding was determined by measuring [^{3}H]ryanodine binding in the presence of a 1000-fold excess of unlabelled ryanodine. All binding assays were performed in duplicate.

RESULTS

Solution changes by stop-flow causes Ca^{2+} release in untransfected HEK-293 cells

We examined whether different protocols for medium changes could influence intracellular Ca^{2+} release in HEK-293 cells. In the protocol used in our laboratory, medium changes were allowed to flow over the cells (over-flow) [3,6,8]; in the protocol of Querfurth et al. [9], flow was interrupted and then medium was removed and replaced with caffeine-containing medium (stop-flow). When we compared these two methods for medium changes, we found marked differences in Ca^{2+} release responses in untransfected HEK-293 cells. In the experiment illustrated in Figure 1, we performed Ca^{2+} imaging on an untransfected HEK-293 cell loaded with fura-2 to measure intracellular Ca^{2+} release responses. Medium changes were performed by either stop-flow or over-flow in the presence or absence of Ca^{2+} in the medium. Ca^{2+} release was induced repetitively in the same cell when the medium was changed by stop-flow, in both the presence and absence of medium Ca^{2+} and in both the presence and absence of 15 mM caffeine. Ca^{2+} release was not induced when medium changes were performed by over-flow. Overall, approx. 35 %

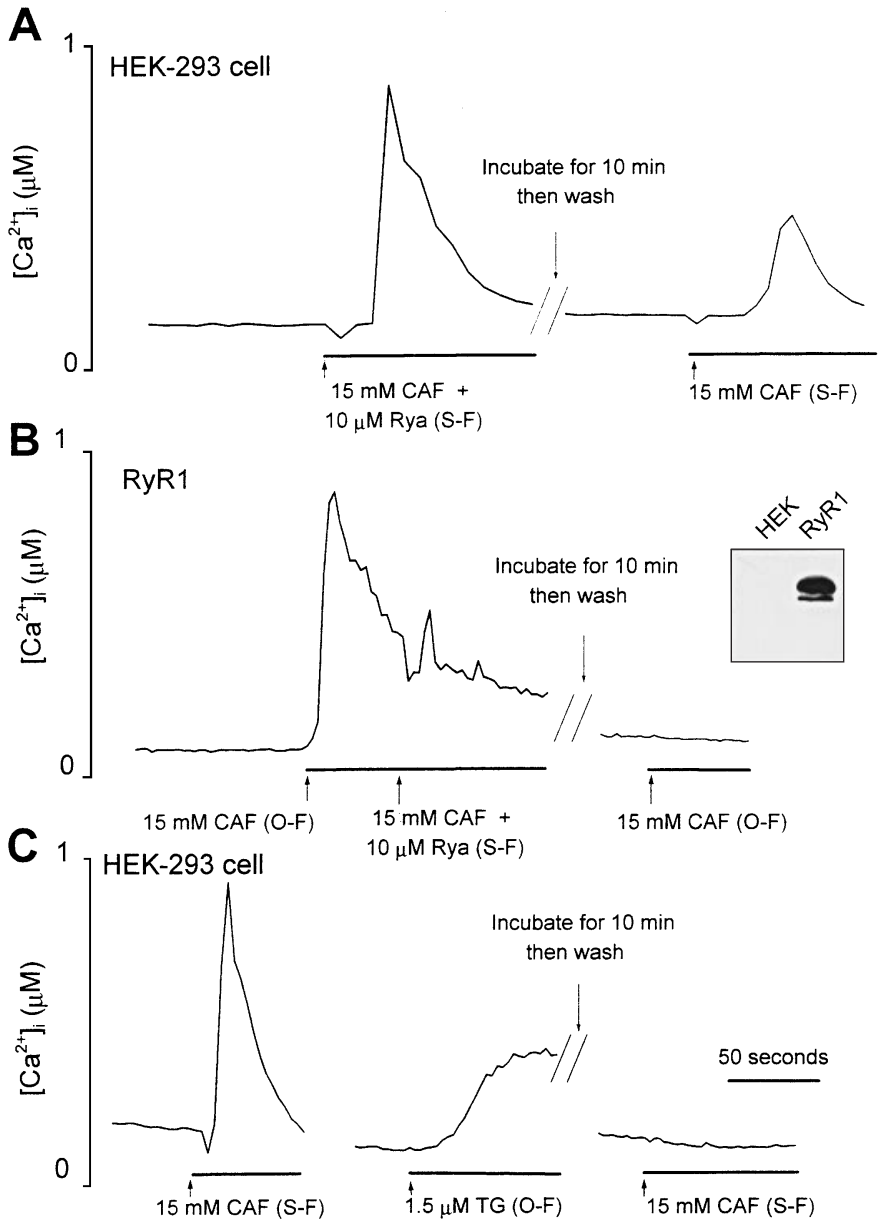

Figure 2 Inhibitory effect of ryanodine and thapsigargin on Ca^{2+} release induced by caffeine added by stop-flow (A, C) and over-flow (B)

(**A**) A single fura-2-loaded HEK-293 cell was treated with 15 mM caffeine (CAF) plus 10 μM ryanodine (Rya) added by stop-flow (S-F). The caffeine response was retained after a 10 min incubation with 10 μM ryanodine, which should have closed an RyR channel. (**B**) A single *RYR1*-transfected cell was treated with 15 mM caffeine added by over-flow (O-F), followed by the addition, by stop-flow, of 15 mM caffeine plus 10 μM ryanodine. The caffeine response was fully inhibited after a 10 min incubation with 10 μM ryanodine. The inset shows a Western blot of whole cell lysates obtained from untransfected (HEK) and *RYR1* cDNA-transfected (RyR1) cells. (**C**) A single untransfected cell was incubated with 15 mM caffeine, added by stop-flow, followed by 1.5 μM thapsigargin (TG). The subsequent response to 15 mM caffeine, added by stop-flow, was inhibited. Thapsigargin had the same inhibitory effect on caffeine-induced Ca^{2+} release in *RYR1*-transfected cells (results not shown). Each Figure is representative of more than 30 out of 50 cells imaged in three different experiments.

(more than 50/150) of non-transfected HEK-293 cells imaged released Ca^{2+} in response to the stop-flow method of medium changes. The same results were obtained when we measured Ca^{2+} release with the HEK-293 cell isolate used in the study of Querfurth et al. [9].

In contrast with the results reported by Querfurth et al. [9], we did not find that Ca^{2+} release was dependent on the presence of 15 mM caffeine in the induction medium. The level of Ca^{2+} release (means $\pm$ S.E.M.) in the absence of caffeine (1120 ± 55 nM, $n = 25$) was approximately the same as the level of Ca^{2+} release in the presence of 15 mM caffeine (1261 ± 82 nM, $n = 30$). Because Ca^{2+} release from non-transfected HEK-293 cells under stop-flow conditions was independent of caffeine, it was impossible to obtain a dose–response curve. It is of interest that Querfurth et al. [9] did not present a dose–response curve for Ca^{2+} release by caffeine, although a dose–response curve was generated with ionophore. Thus, in both studies, the Ca^{2+} release response to solution changes was all or nothing and, in our hands, occurred in both the presence and the absence of caffeine. In contrast, caffeine-induced Ca^{2+} release from *RYR1*-transfected

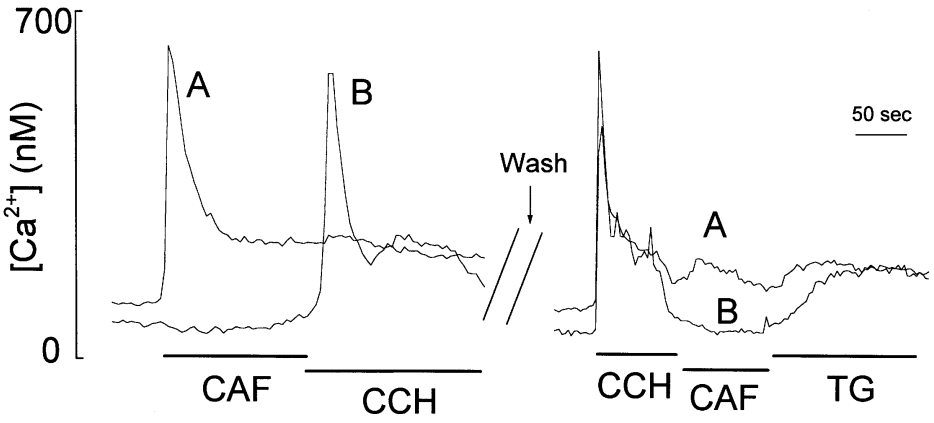

Figure 3 Effects of caffeine, carbachol and thapsigargin on Ca^{2+} release in *RYR1*-transfected and untransfected HEK-293 cells

Two cells from a population of HEK-293 cells transfected with *RYR1* cDNA were treated with reagents by over-flow. Caffeine (CAF, 10 mM) is a specific agonist of RyR; carbachol (CCH, 20 μM) is an agonist of an endogenous muscarinic receptor that releases Ins(1,4,5)P_3, activating an Ins(1,4,5)P_3 receptor Ca^{2+} release channel; thapsigargin (TG, 1.5 μM) is an irreversible SERCA inhibitor. Cell A, deduced to be transfected, released Ca^{2+} in response to both caffeine and carbachol, but did not release Ca^{2+} in response to thapsigargin added in the presence of caffeine, indicating that caffeine had emptied the thapsigargin-sensitive store. Cell B, deduced to be untransfected, released Ca^{2+} in response to carbachol but not caffeine, and released Ca^{2+} in response to thapsigargin added in the presence of caffeine, indicating that caffeine had not emptied the thapsigargin-sensitive store. These results are representative of three independent experiments with more than ten cells per experiment.

HEK-293 cells was dose-dependent when increasing concentrations of caffeine were added by over-flow [3].

Ryanodine does not inhibit Ca^{2+} release from untransfected HEK-293 cells

In Figure 2 we compare the effects of ryanodine and thapsigargin on caffeine-induced Ca^{2+} release under stop-flow and over-flow conditions in untransfected and transfected HEK-293 cells. In untransfected HEK-293 cells, the addition of 10 μM ryanodine in the presence of 15 mM caffeine to induce prolonged inhibition of RyR channels failed to bring about more than a 40% inhibition of subsequent Ca^{2+} release after wash-out and re-exposure to caffeine by stop-flow (Figure 2A). In contrast, the same treatment of HEK-293 cells transfected with *RYR1* led to an almost complete inhibition of subsequent caffeine-induced Ca^{2+} release by over-flow (Figure 2B). It therefore seems unlikely that the Ca^{2+} released from the untransfected HEK-293 cells was through a ryanodine-sensitive channel.

An additional observation was that 1.5 μM thapsigargin, an irreversible sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) inhibitor that elevates cytosolic [Ca^{2+}] by the inhibition of re-uptake, fully inhibited subsequent caffeine-induced Ca^{2+} release, regardless of the way in which caffeine was added (Figure 2C). All of these results indicate that the stop-flow method for changing media causes Ca^{2+} release from a SERCA-dependent Ca^{2+} store in HEK-293 cells but the release does not depend on the activation of an endogenous RyR.

Caffeine and carbachol responses can distinguish between transfected and untransfected HEK-293 cells

In Figure 3 we show that two types of cellular response can be observed in HEK-293 cells that have been subjected to transfection with *RYR1* cDNA. Under our transfection conditions, only 40–60% of HEK-293 cells are transfected and express RyR1; the remainder behave as untransfected cells. Cell A responded to caffeine added by over-flow. In our view, this cell was transfected with *RYR1*, because there was a clear Ca^{2+} release response to caffeine. Carbachol increases intracellular [Ca^{2+}] by the activation of a phospholipase C through an endogenous muscarinic receptor in HEK-293 cells, resulting in the elevation of intracellular [Ins(1,4,5)P_3] and the activation of Ins(1,4,5)P_3 receptors [12]. Cell A did not respond to carbachol added directly after stimulation with caffeine, but the carbachol response was restored when caffeine was washed away. When thapsigargin was added to this cell in the presence of caffeine, no further Ca^{2+} was released, indicating that the Ca^{2+} store was depleted in the presence of caffeine. Cell B did not respond to caffeine added by over-flow. In our view, this cell was untransfected, because its lack of response to caffeine was characteristic of 97/100 untransfected cells described previously [3,8]. Cell B responded to carbachol regardless of whether carbachol was added before or after caffeine. When thapsigargin was added to cell B in the presence of caffeine, a large amount of Ca^{2+} was released, indicating that the Ca^{2+} store had not been depleted in the presence of caffeine. Thus cells A and B, likely to correspond to transfected and untransfected cells in the same population, can be distinguished by the fact that both had carbachol- and thapsigargin-sensitive Ca^{2+} stores, but only one had a caffeine-sensitive Ca^{2+} store, which, we infer, results from its transfection with *RYR1*. Clearly, if all HEK-293 cells expressed high levels of endogenous RyR, as proposed by Querfurth et al. [9], we would not have been able to distinguish between these two types of cells.

[^{3}H]Ryanodine binding

As an additional measurement of the level of expression of endogenous RyR proteins in HEK-293 cells, we performed [^{3}H]-ryanodine binding (Figure 4). In previous studies we measured B_{max} values of 180 fmol/mg of protein for [^{3}H]ryanodine binding to whole cell extracts from *RYR1*-transfected cells and 760 fmol/mg for extracts from *RYR2*-transfected cells. However, we could not measure any specific [^{3}H]ryanodine binding in whole cell lysates [6] or in microsomes [5] isolated from untransfected HEK-293 cells under the same conditions [6]. A typical experiment, in which no measurable [^{3}H]ryanodine binding was observed in the vector-transfected isolate of HEK-293 cells obtained from Dr Haughey, is presented in Figure 4. The results

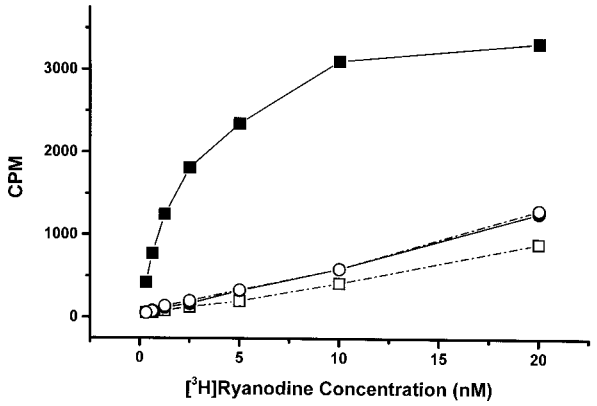

Figure 4 [^{3}H]Ryanodine binding to CHAPS-solubilized lysates from HEK-293 cells transfected with vector or *RYR2* cDNA

CHAPS-solubilized proteins (25 μl) were incubated with 0.31–20 nM [^{3}H]ryanodine in binding buffer (pCa 4) containing 0.1% (w/v) CHAPS and 1 mM ATP at 37 °C for 2 h. [^{3}H]Ryanodine binding was measured by filtration, as described in the Materials and methods section. Symbols: ●, ○, pcDNA-transfected cells; ■, □, *RYR2* cDNA-transfected cells; ●, ■, total [^{3}H]ryanodine binding; ○, □, non-specific [^{3}H]ryanodine binding. The ordinate shows the radioactivity (c.p.m.) bound to the filter in the presence and the absence of a 1000-fold excess of unlabelled ryanodine for both untransfected and transfected HEK-293 cells, rather than the amount of [^{3}H]ryanodine bound, to emphasize the accuracy of our [^{3}H]ryanodine binding measurements. Scatchard analysis yielded a K_d of 2.3 nM and a B_{max} of 1.1 pmol/mg of protein for *RYR2*-transfected cells, but no specific binding for pcDNA-transfected HEK-293 cells. In results for pcDNA-transfected cells, HEK-293 cells obtained from Dr Haughey were used, whereas with data from *RYR2*-transfected cells, HEK-293 cells from our laboratory were used.

presented in Figure 4 are expressed in terms of c.p.m. rather than amount of [^{3}H]ryanodine bound, to emphasize the level of accuracy of our [^{3}H]ryanodine binding measurements. The calculated B_{max} for [^{3}H]ryanodine binding to RyR2 expressed exogenously in the isolate of HEK-293 cells used in our laboratory was 1.1 pmol/mg protein (see the legend to Figure 4). Thus we could not confirm the report by Querfurth et al. [9] of a B_{max} for ryanodine binding of 25 fmol/mg of microsomal protein from HEK-293 cells.

DISCUSSION

The issue of caffeine-sensitivity of intracellular Ca^{2+} stores in non-muscle cells is controversial. Caffeine-induced Ca^{2+} release from intracellular Ca^{2+} stores in untransfected HEK-293 cells was observed by Querfurth et al. [9] but not in our studies [3,5,6,8]. The basis for this discrepancy has been clarified in the present study by our demonstration that stop-flow medium changes alone can induce Ca^{2+} release from intracellular stores in untransfected HEK-293 cells. We have supported this observation by showing that ryanodine does not block stop-flow medium-change-induced Ca^{2+} release in untransfected cells, whereas it does in transfected cells. We have also demonstrated that two types of cell can be distinguished in HEK-293 cell populations in which approx. 40–60% of cells have been transfected. Those proposed to be transfected have caffeine-, carbachol- and thapsigargin-sensitive Ca^{2+} release, whereas those proposed not to be transfected do not have caffeine-sensitive Ca^{2+} release but retain carbachol- and thapsigargin-sensitive Ca^{2+} release. If HEK-293 cells expressed RyR endogenously, it is likely that all cells examined would have demonstrated caffeine-sensitive Ca^{2+} release.

The level of expression of endogenous RyR protein in HEK-293 cells is critical, because this is the cell line of choice for the heterologous expression of RyRs [3,5,6,8]. Here we present further evidence that the levels of endogenously expressed RyR in untransfected HEK-293 cells are very low. Neither Querfurth et al. [9] nor we could detect endogenous RyR proteins in extracts of HEK-293 cells by Western blotting, although we have observed huge amounts of expressed wild-type and mutant RyR1 (Figure 2) [3], RyR2 [6] and RyR3 [2] proteins under comparable conditions. It should be noted that Querfurth et al. [9] could only detect RyR through the highly sensitive immunoprecipitation of radiolabelled cells or by Western blotting of immunoprecipitates. Although Querfurth et al. [9] reported cellular immunostaining, a suitable control for background staining was not provided.

In our single-channel studies with planar lipid bilayers, performed over the past 6 years, we have not encountered the incorporation of any ryanodine- or caffeine-sensitive single-channel activity from extracts of non-transfected HEK-293 cells or any wild-type RyR channel activity from extracts of HEK-293 cells transfected with the Ca^{2+} sensor mutant E3885A [7] or other caffeine-insensitive RyR mutants (results not shown). Thus we have not been able to obtain any evidence for the presence of even low levels of endogenous RyR expression in HEK-293 cells [1,2,7]. This could be because a relatively high concentration of channel protein is required for successful incorporation from bathing media [13]. The single-channel properties of all three RyR isoforms are known. Thus, from a practical point of view, if endogenous RyR channels were to be expressed at low levels in HEK-293 cells, it is unlikely that their functional properties, even if they could be detected in single-channel assays, would be mistaken for mutant channel function.

If RyRs were present at a high level in untransfected HEK-293 cells, caffeine-induced Ca^{2+} release would empty the intracellular Ca^{2+} store as it does in PC12 cells [14] and in *RYR1*-transfected HEK-293 cells. The Ca^{2+} store would not be refilled unless caffeine were removed from the medium (Figure 3) [14]. If the Ca^{2+} store were depleted, it would initiate a programme of events that would lead to apoptosis [15]. Thus if caffeine did cause significant Ca^{2+} release in HEK-293 cells, as Querfurth et al. [9] have reported, most of their HEK-293 cells would have died after incubation in 5 mM caffeine for the 14 h before amyloid-β peptide (Aβ) production was measured [16]. Because we have observed that HEK-293 cells can survive in medium containing 10 mM caffeine for more than 1 month without obvious abnormality (J. Tong and D. H. MacLennan, unpublished work), it is unlikely that functional, endogenous RyR channels that would respond to caffeine are expressed in any significant amount in HEK-293 cells.

Although we do not dispute that very low levels of RyR might be expressed in HEK-293 cells, we do not believe that the activation of endogenous RyR channels resulted in the high level of Ca^{2+} release in the studies of Querfurth et al. [9]. It is possible that the cellular deformation that we observed on stop-flow perfusion might have led to mechanical stress-induced Ca^{2+} entry into the cytoplasm [17]. Ca^{2+} release was observed in only approximately one-third of cells when medium was changed by stop-flow in the present study and in [9]. In some cells, we did not see a response to the first stop-flow medium change but the cell responded to a later medium change. After the first response, the cells seemed to be sensitized to subsequent stop-flow medium changes. This might also have been true of Figure 2(C) in [9].

Recently, RyR gene expression has been found in several non-excitable tissues and cells [18–20]. It is quite possible that very low levels of RyRs might be expressed endogenously in all cells, so that the only practical way to advance our understanding of RyR molecules will be to accommodate these very low levels of

expression. For example, our previous successful characterization of SERCA mutants [21] was performed in microsomal preparations that had a low background of Ca^{2+} transport [22]. With both SERCA and RyR proteins, we have demonstrated that high levels of expressed activity make it fully feasible to accommodate low levels of endogenous activity [1–3,5,6,8]. On this basis, HEK-293 cells provide a valid cell line for the study of exogenous RyR proteins.

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