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Functional expression of the calcium release channel from skeletal muscle ryanodine receptor cDNA

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Combined patch-clamp and fura-2 measurements were performed to study the calcium release properties of Chinese hamster ovary (CHO) cells transfected with the rabbit skeletal muscle ryanodine receptor cDNA carried by an expression vector. Both caffeine (1–50 mM) and ryanodine (100 μM) induced release of calcium from intracellular stores of transformed CHO cells but not from control (non-transfected) CHO cells. The calcium responses to caffeine and ryanodine closely resembled those commonly observed in skeletal muscle. Repetitive applications of caffeine produced characteristic all-or-none rises in intracellular calcium. Inositol 1,4,5-trisphosphate (IP_3) neither activated the ryanodine receptor channel nor interfered with the caffeine-elicited calcium release. These results indicate that functional calcium release channels are formed by expression of the ryanodine receptor cDNA.

cDNA expression; Ryanodine receptor; Calcium release channel; Caffeine; Fura-2; (Chinese hamster ovary cell)

1. INTRODUCTION

The key events in excitation-contraction coupling of skeletal muscle comprise the membrane depolarization of transverse tubules (T tubules) and subsequent release of calcium from the terminal cisternae of the sarcoplasmic reticulum (SR) [1,2]. Calcium release from the SR occurs through a channel which is generally referred to as the ryanodine receptor, since it represents a high-affinity binding site for the plant alkaloid ryanodine. Reconstitution of the purified ryanodine receptor protein in planar lipid bilayers has supported the idea of its functioning as a calcium release channel [3,4]. The properties of the calcium release mechanism have been studied in intact muscle and in 'skinned' muscle fibers, where it was found that calcium release can be induced either depolarization, increases in the concentration of free intracellular calcium $[Ca]_i$ (Ca-induced calcium release) or the pharmacological actions of ryanodine and caffeine (see [1,4] for review). Also, inositol 1,4,5-trisphosphate (IP_3) has been suggested to play a role in excitation-contraction coupling [5–7], possibly by activating the ryanodine receptor channel. However, the in vivo physiological activation of calcium release by IP_3 in skeletal muscle is a matter of debate [8,9].

The ryanodine receptor is thought to form a homotetrameric complex (monomeric $M_r \sim 450\,000$) with the characteristic 'foot' structure which spans the gap between SR and T-tubule membranes, as shown by electron micrographs [10,11]. Cloning and sequence analysis of cDNA have revealed that the rabbit skeletal muscle ryanodine receptor is composed of 5037 amino acids with the C-terminal region which includes 4 putative transmembrane segments, probably forming the calcium release channel, and the remaining portion apparently constituting the 'foot' structure [12]. It has also been shown that expression of the cloned cDNA in Chinese hamster ovary (CHO) cells yields a protein indistinguishable from the ryanodine receptor in immunoreactivity, molecular size and ryanodine binding [12].

We have now carried out combined patch-clamp [13] and fura-2 [14] experiments which show that the skeletal muscle ryanodine receptor expressed in CHO cells indeed functions as an intracellular calcium release channel. Thus, caffeine and ryanodine both release calcium from intracellular stores in a way reminiscent of responses observed in skeletal muscle.

2. MATERIALS AND METHODS

Clones of CHO cells (C7311 and C798) that were transformed with the plasmid pRRS7 to express the rabbit skeletal muscle ryanodine receptor were obtained as described previously [12]. An additional clone (C11727) was isolated by transfecting CHO cells with the plasmid pRRS11, in which the in-frame ATG triplet located 33 base

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Abbreviations: CHO cell, Chinese hamster ovary cell; SR, sarcoplasmic reticulum; $[Ca]_i$, intracellular calcium concentration; IP_3, inositol 1,4,5-trisphosphate; GTPγS, guanosine 5'-*O*-3-thiotriphosphate

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pairs upstream from the translational initiation codon of pRRS7 was deleted. pRRS11 was constructed as follows (see [12] for cDNA clones and nucleotide numbers identifying restriction endonuclease sites). pRR705 was digested with *Sma*I, ligated with the *Hin*dIII linker dACAAGCTTGT and cleaved with *Hin*dIII and *Sph*I. The resulting 0.36-kilobase-pair (kb) *Hin*dIII(linker)/*Sph*I(335 fragment, the *Sph*I(335)/*Bgl*II (1855) fragment from pRR256 and the *Bgl*II(1855)/*Sac*I(3629) fragment from pRR229 were ligated with the 5.6-kb *Sac*I (3629)/*Hin*dIII(vector) fragment from pRRS1b to yield pRRS1c. The 6.5-kb *Hin*dIII(vector)/*Xho*I(6467) fragment from pRRS1c, the *Xho*I(6467)/*Bam*HI(10983) fragment from pRRS2M and the 4.3-kb *Bam*HI(10983)/*Xba*I(vector) fragment from pRRS3 were ligated with the *Xba*I/*Hin*dIII fragment from pSP64 (Promega) to yield pRRS10. The 15.3-kb *Hin*dIII fragment containing the entire protein-coding sequence from pRRS10 was cloned into the *Hin*dIII site of pKNH [15] to yield pRRS11. CHO cells were transfected with *Pvu*I-cleaved pRRS11 and G418-resistant clones were screened by RNA blotting analysis as in [12]. Expression of the ryanodine receptor in C11727 cells was confirmed by immunoblotting analysis and [^{3}H]ryanodine binding assay as in [12]; Scatchard analysis showed that membrane preparations from C11727 cells had a ryanodine binding capacity (B_{max}) of 3.3 pmol/mg protein and a binding affinity (K_d) of 20 nM.

Most of the calcium release experiments were performed with clone C7311. The percentage of cells that responded to caffeine gradually decreased over 2–3 weeks in the course of cell growth. Therefore, a new batch of frozen cells was cultured every two weeks. Round cells with refractile inclusions were selected for the experiments. Normal and transformed CHO cells were plated on cover slips and cultured at 37°C and 10% CO_2 in a medium supplemented with fetal calf serum (10%), glutamine (2 mM), penicillin (0.06 mg/ml), streptomycin (0.11 mg/ml) and Na-pyruvate (1 mM). Experiments were performed at 23–26°C in a saline solution containing (in mM): NaCl 140, KCl 2.8, $CaCl_2$ 1, $MgCl_2$ 2, glucose 12, Hepes-NaOH 10, pH 7.2. Calcium measurements and calibration procedures were performed as described previously [14,16,17]. Briefly, fura-2 fluorescence was excited at 360 and 390 nm and $[Ca]_i$ was calculated from the fluorescence ratio. Data points were sampled at 500 ms intervals with a computer calculating $[Ca]_i$ on-line. Two methods of loading the calcium indicator dye fura-2 were used: (1) Ester-loading: cells were incubated at room temperature in standard saline with fura-2-AM (1–2 μM) for 30 min after which they were washed and placed in an incubator for 15 min at 37°C. (2) Pipette-loading: fura-2 pentapotassium salt (100 μM) was added to the internal solution used for filling patch-pipettes. The pipette filling solution comprised (in mM): K-glutamate 145, NaCl 8, $MgCl_2$ 1, Mg-ATP 0.5, Hepes-KOH 10, pH 7.2. Drug applications were made by pressure-ejection from a pipette containing extracellular saline and the compound under investigation. Caffeine (Sigma) was used at concentrations of 1–50 mM, ryanodine (HPLC Grade, Wako Ltd., Japan) at 100 μM, and procaine (Sigma) at 1–10 mM. IP_3 (Amersham) at 10 μM or GTPγS (kindly provided by Dr F. Eckstein, Göttingen, FRG) at 100 μM was added to the intracellular solution.

3. RESULTS

The first series of experiments was designed to find out whether CHO cells transformed to express the rabbit skeletal muscle ryanodine receptor have functional calcium release channels. Fig.1A illustrates typical changes in $[Ca]_i$ in response to a brief application of caffeine (10 mM). Similar responses to caffeine (10–50 mM) were observed in the 14 out of 24 cells tested (clone C7311). Cells from clone C11727 were similarly responsive (7 out of 11 cells), whereas cells from clone C798 responded less frequently (8 out of 29 cells). In non-transfected CHO cells application of 10 mM caffeine never elicited measurable calcium changes (19 cells). Caffeine at 1 mM yielded indistinguishable changes in $[Ca]_i$ but the percentage of responsive cells was slightly lower (5 out of 11 cells from clone C7311). Subsequent experiments were therefore carried out at a caffeine concentration of 10 mM.

The time course of $[Ca]_i$ responses to caffeine was relatively uniform, with the rising phase reaching a peak within 2–6 s. The peak amplitudes of the responses were unrelated to the concentration of caffeine used and averaged 0.69 ± 0.24 μM (mean ± SD, n = 24, excluding unresponsive cells). The delay between application of caffeine and onset of the $[Ca]_i$ response showed some degree of variability. In most cases the $[Ca]_i$ response occured within 2–10 s but in some cells it took more than 60 s before $[Ca]_i$ started to rise. Following the rise, $[Ca]_i$ remained elevated for 10–40 s and then declined precipitously regardless of whether the caffeine application continued or had been stopped.

The changes in $[Ca]_i$ were clearly due to release of calcium from intracellular stores, since similar responses were observed after removing calcium from the bath solution (fig.1B). Further evidence for this conclusion was provided by experiments in which caffeine was applied repeatedly to test for possible refilling of the internal calcium pools (fig.1C). Repetitive responses were elicited by stimulation at 1 min intervals. In this case, however, after 4 applications the fifth one was ineffective in inducing calcium release. Subsequent applications produced either normal-sized responses or failures, presumably depending on the filling state of the internal stores. The responses seemed to be 'all-or-none' and a similar behaviour was observed in a number of cells. This pattern may have important implications for the regulation of the calcium release mechanism (see section 4). Some cells, however, remained refractory to further applications of caffeine once the stores had been depleted.

Ryanodine is another substance known to release calcium from skeletal muscle SR [18] and it proved to do so in transformed CHO cells. We observed quite variable responses upon application of ryanodine (100 μM). Examples of such experiments are illustrated in fig.2A. Ryanodine responses were usually very sluggish and outlasted the actual drug application time. Slow and multiphasic calcium transients like those in the middle trace of fig.2A were often superimposed on a slower wave of $[Ca]_i$. The rather slow onset of the response probably cannot be ascribed to ryanodine not reaching its site of action, since applying ryanodine intracellularly through patch-pipettes yielded similarly slow and long-lasting changes in $[Ca]_i$ (not illustrated).

We have tested 3 additional ways of activating the calcium release mechanism, which all proved to be ineffective. (1) We were unable to induce changes in $[Ca]_i$ following depolarization of the plasma membrane of

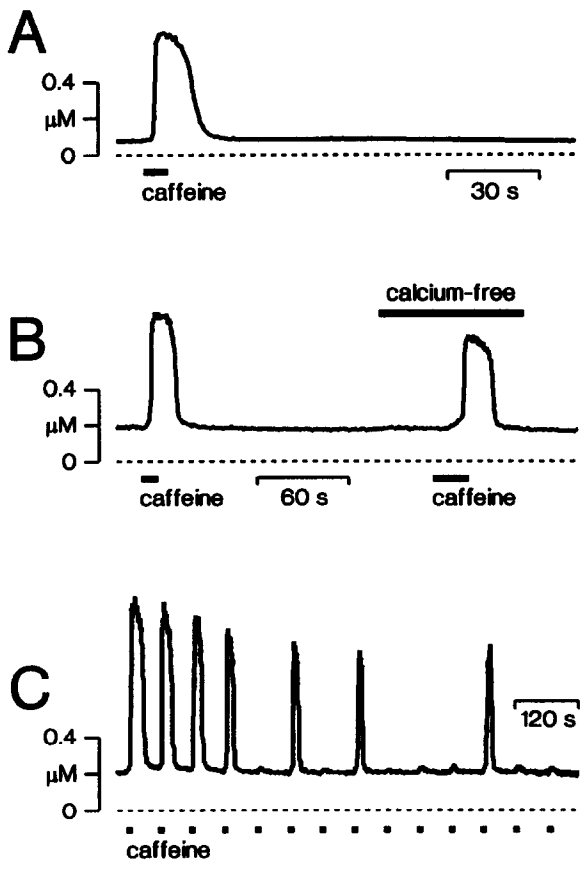

Fig.1. Caffeine-induced release of calcium from CHO cells transformed to express the ryanodine receptor. (A) Typical response of a fura-2-AM-loaded cell (clone C7311) to caffeine (10 mM). Application of caffeine was performed by pressure-ejection from a wide-tipped micropipette for the time indicated by the bar. (B) Experimental conditions as in A, except that the second application of caffeine was performed after replacing the normal saline by one containing nominally zero calcium (as indicated in the trace). The application pipette contained 10 mM caffeine in calcium-free saline (for both applications). (C) Experimental conditions as in A, except that the cell was repeatedly subjected to caffeine pulses of 10 s duration at 1 min intervals (as indicated in the trace). The baselines are indicated by broken lines.

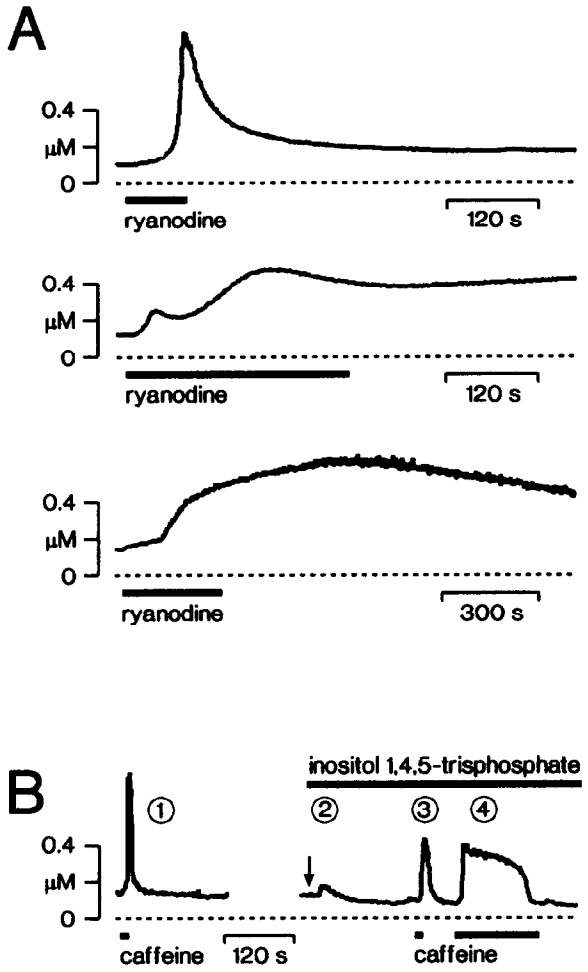

Fig.2. Changes in $[Ca]_i$ induced by ryanodine, caffeine and IP_3. In all cases, transformed CHO cells (clone C7311) were used and periods of drug application are indicated by bars. (A) Examples of responses of fura-2-AM-loaded cells to ryanodine (100 μM). Application of ryanodine was performed by pressure-ejection from a wide-tipped micropipette. Note the different time scales. (B) Effects of IP_3 (10 μM) and caffeine (10 mM). The cell was loaded with fura-2-AM and tested for caffeine response (1). Then a whole-cell recording configuration was established (arrow) with a patch-pipette containing fura-2 (100 μM) and IP_3 (10 μM). Note the small calcium transient induced by IP_3 (2). Subsequent applications of caffeine (3 and 4) produced large changes in $[Ca]_i$. The trace is interrupted during the time of approaching the cell with the patch-pipette. The baselines are marked by broken lines.

transformed CHO cells under voltage clamp. (2) Perfusion of CHO cells with IP_3, which has been invoked as a candidate for activating calcium release from SR [5,6], did not stimulate the caffeine-sensitive calcium release mechanism. We were surprised to learn that IP_3, which causes calcium release in most cell types, induced only very small changes in $[Ca]_i$ in transformed CHO cells. In no case did we see calcium release as dramatic as that found with caffeine. Fig.2B shows an experiment in which we first induced calcium release by caffeine to make sure that the cell used was responsive, and then the cell was dialysed with a patch-pipette containing 10 μM IP_3. After establishment of the whole-cell configuration, IP_3 induced a small calcium transient and subsequent applications of caffeine caused considerably larger changes in $[Ca]_i$ despite the presence of IP_3. (3) Perfusing CHO cells with GTPγS (100 μM) to activate G proteins gave results similar to those obtained with IP_3, i.e. there was no or very little calcium releasing activity, the latter being induced probably by liberation of IP_3. Caffeine was still able to release a large amount of calcium from internal stores in cells perfused with GTPγS (data not shown).

Further attempts to characterize the expressed ryanodine receptor, particularly with respect to pharmacological blocking agents such as dantrolene or ruthenium red, were precluded by their interference with fura-2 fluorescence. Unfortunately, CHO cells

also lack calcium-activated potassium or chloride channels, which could have been used as an alternative way of monitoring changes in $[Ca]_i$. Procaine, another blocker of calcium release in skeletal muscle [1], was not very effective in suppressing caffeine-induced calcium release. Procaine at 1 mM showed no obvious effect on caffeine responses, although very high concentrations (10 mM) were effective in most cells.

4. DISCUSSION

The present results unequivocally show that the ryanodine receptor of skeletal muscle functions as a calcium release channel when expressed in CHO cells by transformation with its cDNA. The pharmacological activation of the calcium release mechanism is similar to that observed in intact skeletal muscle fibres [19], as witnessed by the changes in $[Ca]_i$ induced by caffeine and ryanodine. As in muscle cells, caffeine causes a prompt increase in $[Ca]_i$ that rapidly fades as the drug is removed. In contrast, ryanodine produces a slower but sustained increase in $[Ca]_i$ that persists long after drug removal, again in agreement with studies on the native tissue. This behavior of ryanodine is consistent with the observation that it induces a persistent, but partial activation of the ryanodine receptor channel reconstituted in planar lipid bilayers [20,21].

Multiple applications of caffeine at regular intervals produced an interesting response pattern in many of the cells tested. The first few changes in $[Ca]_i$ were quite uniform in shape and amplitude, whereas subsequent applications resulted in either complete failures or normal-sized $[Ca]_i$ responses, rather than in gradual decreases in the amplitude of the responses. This behaviour might be interpreted by a mechanism in which the activation of the calcium release channel by caffeine is dependent on the filling state of the intracellular stores [1]. In such a model ineffective stimuli should not lead to the opening of the calcium release channel due to a low filling state of the intracellular stores. However, refilling would continue so that the stores are ready for release at the time of the next stimulus. The hypothesis that a calcium release channel is sensitive to the intrastore calcium concentration has been explored in theoretical models for IP_3-induced calcium release [22] and for Ca-induced calcium release from heart SR [23].

Our finding that in transformed CHO cells IP_3 hardly increases $[Ca]_i$ does not support the hypothesis that IP_3 is an activator of the ryanodine receptor channel. The fact that caffeine, applied after dialysing cells with IP_3, can elicit a large calcium signal suggests that the caffeine-sensitive calcium pool in these cells is different from and larger than the IP_3-sensitive pool. It is known that different fractions of the endoplasmic reticulum show different calcium releasing activities in response to IP_3 [24], and it may be suggested that the expressed ryanodine receptor is incorporated into parts of the endoplasmic reticulum that are insensitive to IP_3.

Depolarization and charge movement within the T-tubular membrane are thought to be triggering steps for calcium release in skeletal muscle [25], but we found no depolarization-induced changes in $[Ca]_i$. This implies that the voltage-sensing link between plasma membrane and intracellular stores is missing in CHO cells, where no such voltage sensor is expected. It has been suggested that in the triads of skeletal muscle this link is provided by the dihydropyridine receptor [26–28]. An alternative explanation for the ineffectiveness of depolarization in activating the calcium release mechanism could be that CHO cells lack the specialized morphological organization at the triads of skeletal muscle and that such an organization is required for voltage-induced activation of the ryanodine receptor channel.

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REFERENCES

[1] Endo, M. (1977) Physiol. Rev. 57, 71–108.
[2] Martonosi, A.N. (1984) Physiol. Rev. 64, 1240–1320.
[3] Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q. and Meissner, G. (1988) Nature 331, 315–319.
[4] Fill, M. and Coronado, R. (1988) Trends Neurosci. 11, 453–457.
[5] Vergara, J., Tsien, R.Y. and Delay, M. (1985) Proc. Natl. Acad. Sci. USA 82, 6352–6356.
[6] Volpe, P., Salviati, G., Di Virgilio, F. and Pozzan, T. (1985) Nature 316, 347–349.
[7] Donaldson, S.K., Goldberg, N.D., Walseth, T.F. and Huetteman, D.A. (1988) Proc. Natl. Acad. Sci. USA 85, 5749–5753.
[8] Walker, J.W., Somlyo, A.V., Goldman, Y.E., Somlyo, A.P. and Trentham, D.R. (1987) Nature 327, 249–252.
[9] Ehrlich, B.E. and Watras, J. (1988) Nature 336, 583–586.
[10] Franzini-Armstrong, C. (1970) J. Cell Biol. 47, 488–499.
[11] Wagenknecht, T., Grassucci, R., Frank, J., Saito, A., Inui, M. and Fleischer, S. (1989) Nature 338, 167–170.
[12] Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T. and Numa, S. (1989) Nature 339, 439–445.
[13] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers Arch. 391, 85–100.
[14] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[15] Fukuda, K., Higashida, H., Kubo, T., Maeda, A., Akiba, I., Bujo, H., Mishina, M. and Numa, S. (1988) Nature 335, 355–358.
[16] Neher, E. (1989) in: Neuromuscular Junction (Sellin, L.C., Libelius, R. and Thesleff, S. eds) pp. 65–75, Elsevier, Amsterdam.
[17] Neher, E., Marty, A., Fukuda, K., Kubo, T. and Numa, S. (1988) FEBS Lett. 240, 88–94.
[18] Meissner, G. (1986) J. Biol. Chem. 261, 6300–6306.
[19] Lüttgau, H.C. and Oetliker, H. (1968) J. Physiol. 194, 51–74.
[20] Imagawa, T., Smith, J.S., Coronado, R. and Campbell, K.P. (1987) J. Biol. Chem. 262, 16636–16643.

[21] Rousseau, E., Smith, J.S. and Meissner, G. (1987) Am. J. Physiol. 253, C364–C368.
[22] Meyer, T. and Stryer, L. (1988) Proc. Natl. Acad. Sci. USA 85, 5051–5055.
[23] Wong, A.Y.K., Fabiato, A. and Bassingthwaighte, J.B. in: Activation, Metabolism and Perfusion of the Heart (Sideman, S. and Beyar, R. eds) pp. 281–295, Nijhoff, Dordrecht.
[24] Henne, V., Piiper, A. and Söling, H.-D. (1987) FEBS Lett. 218, 153–158.
[25] Schneider, M.F. and Chandler, W.K. (1973) Nature 242, 244–246.
[26] Rios, E. and Brum, G. (1987) Nature 325, 717–720.
[27] Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. and Numa, S. (1987) Nature 328, 313–318.
[28] Tanabe, T., Beam, K.G., Powell, J.A. and Numa, S. (1988) Nature 336, 134–139.