Identification of functional type 1 ryanodine receptors in mouse dendritic cells

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Abstract **Ca^{2+} signaling plays an important role in the function of dendritic cells (DC), the specialized antigen-presenting cells of the immune system. Here we describe functional ryanodine receptor (RyR) Ca^{2+} release channels in murine, bone marrow-derived DC. RT-PCR analysis identified selective expression of the type 1 RyR, with higher levels detected in immature rather than mature DC. The RyR activators caffeine, FK506, ryanodine and 4-chloro-*m*-cresol mobilized Ca^{2+} in DC, and responses to 4-chloro-*m*-cresol were inhibited by dantrolene. Furthermore, activation of RyRs both inhibited subsequent inositol trisphosphate-mediated Ca^{2+} release and provoked store-operated Ca^{2+} entry, suggesting a functional interaction between these intracellular Ca^{2+} channels. Thus, the RyR1 channel may play an intrinsic role in Ca^{2+} signaling in DC.**

Key words: Dendritic cell; Calcium; Ryanodine receptor; FK506; Caffeine

1. Introduction

The ryanodine receptor (RyR) is a major intracellular Ca^{2+} release channel found in many cell types. Although the best-known function of RyRs is to provide the Ca^{2+} trigger for muscle contraction the channels may also play important roles in diverse cell signaling pathways [1–3]. Three types of RyR (RyR1–3) have been described; each is the product of a different gene. RyR1 and RyR2 are expressed predominantly in skeletal muscle and heart respectively, although low-level expression is found in a number of tissues. RyR3, in contrast, is widely expressed, including neurons and leukocytes. The role of RyRs in 'non-excitable' lymphoid tissue is not well understood, but RyRs in these cells may serve to amplify Ca^{2+} rises initiated by inositol trisphosphate receptors (IP_3Rs). Indeed, in T cells, RyR3 may contribute to the sustained Ca^{2+} rise that is necessary for T cell proliferation [4,5]. Here we report the expression of the 'skeletal muscle' RyR1 channel in dendritic cells (DC), rare leukocytes highly specialized for the induction of primary immune responses. Immature DC, present in virtually all tissues and organs, possess a high capacity for the uptake and processing of antigen. After encountering antigen these DC migrate to secondary lymphoid tissues where they efficiently stimulate naive antigen-specific T cells [6,7]. Although Ca^{2+} signaling pathways are thought to regulate DC activity [8–10], these pathways have not been well characterized. Here we show that DC express functional RyR1 channels. In addition, we demonstrate that RyR expression is developmentally regulated and that RyRs may functionally interact with the IP_3R Ca^{2+} stores. These results suggest that RyR1 channels are fundamentally involved in DC Ca^{2+} signaling and support a broader role for the RyR1 channel outside skeletal muscle.

2. Materials and methods

2.1. DC culture and purification

DC were cultured from bone marrow cells isolated from the femurs and tibias of normal C57BL/10J mice (6–12 weeks old) using granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin (IL) 4 (4 ng/ml and 1000 U/ml respectively) as previously described [11]. The culture medium, RPMI 1640 (Life Technologies, Rockville, MD, USA), was supplemented with 10% fetal calf serum (Nalgene, Miami, FL, USA), non-essential amino acids, L-glutamine, sodium pyruvate, penicillin–streptomycin and 2-ME (all from Life Technologies). DC were harvested after 5 days of culture and purified by density (145 mg/ml metrizamide; Sigma) centrifugation. For analysis of RyR transcript expression by immature and mature DC, bone marrow cultures were enriched for DC by density centrifugation (165 mg/ml metrizamide) and stained with anti-CD11c and anti-CD86 conjugated to fluorescein isothiocyanate and phycoerythrin respectively (BD Biosciences, San Diego, CA, USA). $CD11c^+$ $CD86^-$ (immature) and $CD11c^+$ $CD86^+$ (mature) cells with high forward and side scatter profiles were separated and purified to $\geq 95\%$ purity using a Beckman Coulter EPICS Elite (Hialeah, FL, USA).

2.2. RT-PCR

Total cellular RNA was extracted using TRIzol (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. First strand cDNA was synthesized using Advantage RT-for-PCR (Clontech Laboratories, Palo Alto, CA, USA) with the supplied $oligo(dT)_{16}$ primer. 50–100 ng of cDNA was used for each subsequent PCR. Relatively equal quantities of cDNA were ensured by amplifying a 540 bp segment of β-actin [12] using a Gene Amp® PCR System 2400 (Perkin Elmer, Norwalk, CT, USA). RyR expression was detected by amplification of a 1.2 kb fragment common to RyR1–3 transcripts [4]. RyR subtype expression was determined by amplifying fragments specific for RyR1 (298 bp), RyR2 (1179 bp) and RyR3 (537 bp) transcripts using subtype-specific primers [13]. Transcripts were amplified by 35 or 40 cycles (RyR1–3 subtypes or pan-RyR, respectively) of 20 s 95°C, 30 s 55°C and 30 s 72°C followed by a final extension step of 5 min at 72°C. RT-PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide fluorescence.

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2.3. Fluorescence measurements

DC were plated on coverslips in culture media and loaded with Fluo-3AM (3–5 μM) for 20 min at 25°C. Cells were then washed with several volumes of bathing solution and left for a further 20 min prior to recording. Standard bathing solution was (in mM) 130 NaCl, 4 KCl, 10 HEPES, 10 glucose, 2 $CaCl_2$, 1 $MgCl_2$, pH 7.3. Drugs were applied via a puffer pipette placed near the cell. Fluorescence measurements were made using a Deltascan fluorometer (Photon Technology International, South Brunswick, NJ, USA) coupled to a Diastar microscope (Reichert Jung) or a Fluoview confocal microscope. Fluo-3 was excited at 488 nm and emitted fluorescence was filtered with a 535 ± 25 nm bandpass filter.

All values are expressed as mean ± S.E.M. Statistical differences were evaluated using Student's *t*-test. Recombinant (r) GM-CSF and IL-4 were gifts of Dr. S.K. Narula (Schering-Plough, Kenilworth, NJ, USA). FK506 was a gift from Fujisawa. Ryanodine was obtained from Calbiochem. All other drugs/reagents were from Sigma.

3. Results

3.1. RyR ligands induce intracellular Ca^{2+} rises in dendritic cells

We tested for the presence of functional RyRs in mature DC ($CD11c^+$, $CD86^+$) using Ca^{2+} imaging techniques. Fig. 1 shows intracellular Ca^{2+} changes (assessed as changes in Fluo-3 fluorescence) in individual cells in response to well-known RyR ligands. One second pulses of caffeine (10 mM) produced rapid and reproducible Ca^{2+} rises (5/6 cells). Ryanodine, the definitive RyR modulator [14], induced either a slow Ca^{2+} transient (50–500 μM, 16/20 cells), or a more gradual Ca^{2+} rise (50 μM, 6/6 cells, data not shown). The immunosuppressant, FK506 (25 μM), induced a slow irreversible Ca^{2+} rise in all cells tested ($n = 5$). This action of FK506 is consistent with its ability to activate RyRs by dissociating the tightly bound FK506 binding proteins from the channel complex [15,16]. The RyR activator, 4-chloro-*m*-cresol (300 μM) [17,18], evoked a large Ca^{2+} rise ($n = 20$), and this effect was prevented by pretreatment with the RyR antagonist dantrolene [19,20] (25 μM, $n = 10$, data not shown). These results provide clear pharmacological evidence for expression of functional RyR channels in DC.

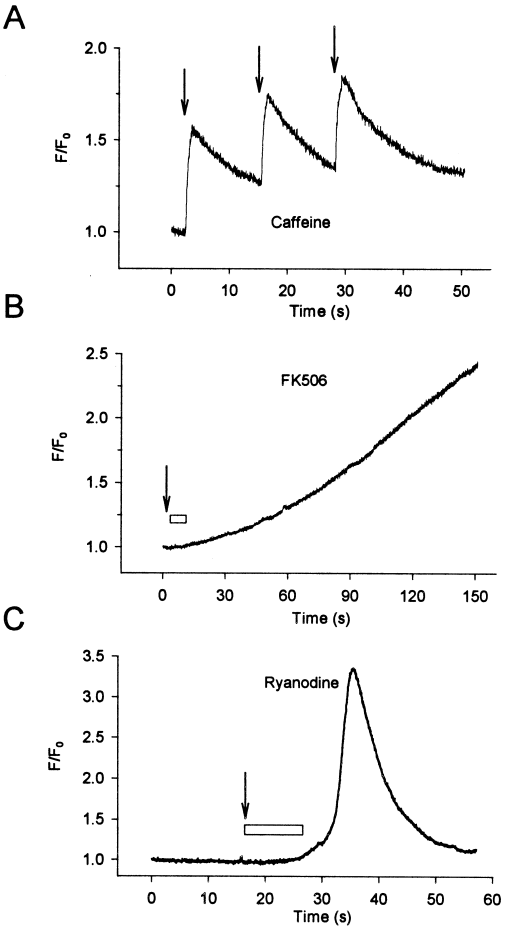

Fig. 1. RyR ligands evoke intracellular Ca^{2+} increases in mature DC. Individual Fluo-3-loaded DC were challenged with the drug-containing solution, applied via a picospritzer pipette. A: One second applications of 10 mM caffeine evoked repetitive Ca^{2+} increases. B: 25 μM FK506 produced a slow irreversible Ca^{2+} rise. C: 50 μM ryanodine produced a slow Ca^{2+} transient.

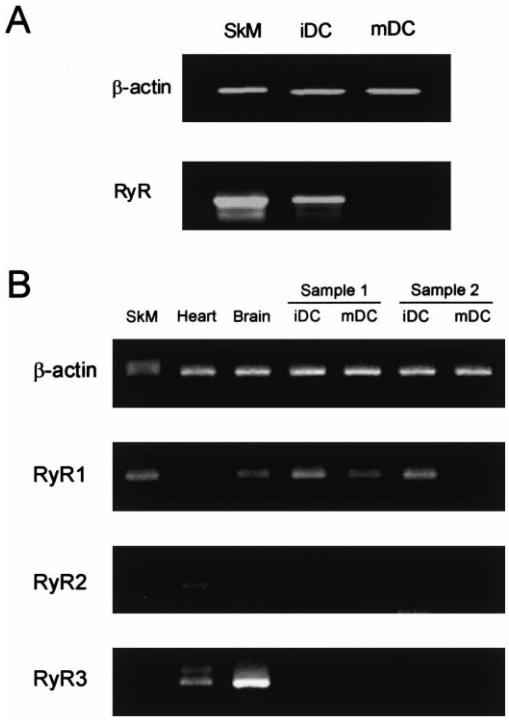

Fig. 2. Identification of RyR1 in DC by RT-PCR. A: Detection of RyR expression in skeletal muscle, immature and mature DC cDNA samples by amplification of a sequence common to all three RyRs. B: RT-PCR of sequences specific for each of the three RyRs (RyR1–3) demonstrates that RyR1 is the predominant subtype expressed in both immature and mature DC. Skeletal muscle, heart, and brain samples were used as positive controls for RyR1–3 expression respectively. Note that a band corresponding to RyR2 from heart cDNA was clearly seen in the original photograph but is not clear in the reproduction. Amplification of β-actin was used as an internal positive control in all experiments.

3.2. Identification of RyR1 in DC by RT-PCR analysis

The expression of RyR gene transcripts was confirmed in DC by amplifying a 1.2 kb sequence common to all RyR subtypes [4] by RT-PCR (Fig. 2A). Relatively high levels of RyR transcripts were identified in both skeletal muscle and immature DC. In contrast, RyR transcripts were barely detectable in mature DC. To identify the RyR, transcripts specific to each RyR subtype were amplified using subtype-specific primers [13]. cDNA samples from skeletal muscle, heart, and brain tissue were amplified in parallel with DC cDNA samples as controls for RyR1, RyR2 and RyR3 respectively.

Fig. 2B shows that only RyR1 transcripts were detected in two samples of both immature and mature DC cDNA. Thus, DC express the type 1 or skeletal muscle RyR, but little or no RyR2 or RyR3. Interestingly, expression of RyR1 transcripts was greater in immature compared with mature DC, consistent with the results found using the pan-RyR primer set (Fig. 2A).

3.3. RyRs are functionally coupled to IP_3R Ca^{2+} stores

We next considered whether RyRs might interact with the IP_3 signaling pathway in DC. IP_3 is a key regulator of $[Ca^{2+}]_i$ in leukocytes [21]; and IP_3-mediated Ca^{2+} rises in DC accompany chemokine and cytokine stimulation. RyRs, in turn, are activated by increases in $[Ca^{2+}]_i$ (a process termed Ca^{2+}-induced Ca^{2+} release). Thus, the initial Ca^{2+} rise triggered by IP_3 could activate RyRs in DC. Such a mechanism would be enhanced if RyRs and IP_3Rs were in close proximity. Although IP_3Rs and RyRs are traditionally thought to utilize separate Ca^{2+} stores there is evidence that these channels may share Ca^{2+} stores in Purkinje neurons [22]. We tested this hypothesis in DC by examining whether activation of RyRs and depletion of the RyR-specific Ca^{2+} store affected subsequent IP_3-mediated Ca^{2+} release. DC were stimulated with 20 μM ATP to evoke IP_3-mediated Ca^{2+} responses as described previously [10,23]. Fig. 3 shows representative ATP-evoked Ca^{2+} transients in individual DC either under control conditions or after treatment with 50 μM ryanodine (10 min). The Ca^{2+} rise in the ryanodine-treated cells was significantly smaller ($P < 0.05$, $n = 4$). This decrease was unlikely due to inhibition of RyRs by ryanodine; Ca^{2+} rises could still be evoked by other RyR ligands after ryanodine treatment, suggesting that RyR channels were not blocked (data not shown). Thus, the depression of the ATP-evoked response by ryanodine is likely due to depletion of the IP_3R-coupled Ca^{2+} store. This suggests that RyRs and IP_3Rs in DC are both expressed in the same membrane with a contiguous Ca^{2+} store. To test this possibility further, we investigated whether RyR activation could lead to activation of store-operated Ca^{2+} entry. We have previously shown in DC that depletion of IP_3R-coupled Ca^{2+} stores by thapsigargin, IP_3, or ATP induces activation of the store-operated current, I_{CRAC} [10]. Therefore, we predicted that depletion of the same store by RyR agonists would also activate I_{CRAC}. In Ca^{2+} imaging experiments DC were treated with caffeine in zero Ca^{2+} medium. Upon readdition of a Ca^{2+}-containing solution, a large Ca^{2+} rise was observed consistent with activation of store-operated channels (data not shown). Thus, in DC both RyRs and IP_3Rs appear to be functionally coupled to I_{CRAC}.

4. Discussion

Our results demonstrate clear evidence for RyR channels in DC. Robust Ca^{2+} rises were observed in response to a number of characteristic RyR ligands. RT-PCR analysis confirmed specific expression of the RyR1 subtype. This result is consistent with the intracellular Ca^{2+} responses. FK506 has more potent effects on RyR1 than the other RyR subtypes [24,25], while dantrolene inhibits RyR1 and RyR3 channels but has no effect on RyR2 [20]. The RyR1 subtype has been traditionally considered a 'skeletal muscle' channel and is essential for triggering muscle contraction [2]. Thus, it is somewhat surprising to find it expressed in DC. RyR1 expression has been reported in B lymphocytes [26,27]. However, the role of this RyR in B cells is unclear; the functional responses are not as marked as we show here in DC. In particular, caffeine and ryanodine fail to elicit Ca^{2+} increases in B cells. In contrast, the RyR3 channel expressed in T cells is activated by cyclic ADP ribose and is believed to play an important role in antigen-stimulated T cell proliferation [5]. DC, in turn, are potent antigen-presenting cells, and the only leukocytes capable of efficiently activating naive T cells. Thus, these data support a role for specific RyR subtypes in different arms of the adaptive immune system.

Interestingly RT-PCR showed that RyR (which is predom-

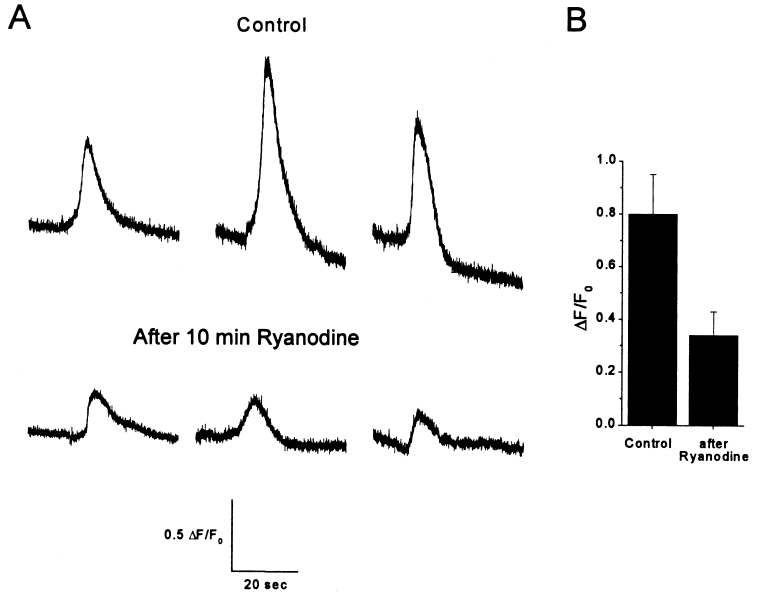

Fig. 3. Ryanodine reduces IP_3R-mediated Ca^{2+} transients. A: Representative IP_3R-mediated Ca^{2+} transients evoked by ATP (30 μM) before and 10 min after treatment with ryanodine (50 μM). Each trace is from a different cell. B: Mean ± S.E.M. response of control and ryanodine-treated groups ($n = 4$ for both, $P < 0.05$).

inantly RyR1) expression declined during DC maturation. Although inducible RyR expression has been reported in many tissues, for example, transforming growth factor-β increases RyR1 and RyR3 mRNA levels in HeLa cells [28], the down-regulation of RyR1 expression is less common. The predominant expression of RyR1 in immature DC may indicate a specific functional role for this channel in DC maturation or migration. However, the significance of this is unclear as following activation, migration to secondary lymphoid tissue and terminal maturation, DC are lost within 24–48 h [29] presumably via programmed cell death [30,31]. Therefore, it is possible that down-regulation of RyR1 mRNA is merely an early manifestation of cell function shutdown prior to apoptosis. Note that functional RyR responses were seen in mature DC (Fig. 1), indicating that RyR protein was still present in these cells. This presumably reflects a slow turnover rate of the channels. Future experiments detailing RyR1 protein expression in DC cultured with survival factors, such as CD40L, should resolve these issues.

Our data suggest that IP_3Rs and RyRs in DC share the same intracellular Ca^{2+} store. These results suggest a functional interplay between IP_3Rs and RyRs during DC Ca^{2+} signaling. A similar functional colocalization of these channels has been proposed for Purkinje neurons [22,32]. Many of the chemokines and growth factors that control DC maturation and migration act via G protein-coupled receptors and initiate IP_3-mediated Ca^{2+} rises [33–36]. Thus, RyRs coupled to IP_3R Ca^{2+} stores may also participate in these important signaling processes. These RyRs may serve to amplify an IP_3-mediated Ca^{2+} spike. Moreover, colocalization of the channels may enhance RyR activation. In muscle, the Ca^{2+} threshold for RyR activation is $\sim$100 nM and maximal activation is seen at $\sim$10–100 μM [1]. The bulk $[Ca^{2+}]_i$ in DC during stimulation may never exceed $\sim$1 μM [10,23], however the $[Ca^{2+}]$ near IP_3R release sites would be much higher. Thus, RyRs may be more effectively activated situated close to IP_3Rs. Activation of RyRs and depletion of the Ca^{2+} store will enhance the activation of the store-operated current, I_{CRAC}. In turn, the sustained Ca^{2+} rise mediated by I_{CRAC} can accelerate DC maturation [10].

In summary, we show that DC express functional type 1 RyRs and propose that these channels may play a developmental-specific role in DC biology.

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