Pharmacology of Ca^{2+} release from red beet microsomes suggests the presence of ryanodine receptor homologs in higher plants

Shelagh R. Muir*, Dale Sanders

The Plant Laboratory, University of York, PO Box 373, York YO1 5YW, UK

Received 28 July 1996; revised version received 28 August 1996

Abstract Cyclic ADP-ribose (cADPR) is known to release Ca^{2+} from plant vacuoles, implying that this NAD^+ metabolite may possess a second messenger role in plants. The degree to which the plant cADPR-gated Ca^{2+} release mechanism resembles cADPR action in animals has been evaluated. cADPR-elicited Ca^{2+} release from red beet microsomes was inhibited by 1 mM procaine but insensitive to heparin. Furthermore, pre-release of Ca^{2+} from red beet vesicles by either 5 mM caffeine or micromolar levels of ryanodine precluded further Ca^{2+} mobilisation by cADPR. Thus, this study argues strongly for conservation between the plant and animal cADPR-elicited Ca^{2+} release mechanisms.

Key words: Calcium; Cyclic ADP-ribose; Ryanodine; Microsome; *Beta vulgaris*

1. Introduction

Changes in cytosolic free Ca^{2+} concentration are now recognised as being crucial in the transduction of a wide variety of stimuli in plant cells [1]. It has been established that inositol 1,4,5-trisphosphate ($InsP_3$) can elicit increases in cytoplasmic Ca^{2+} concentration in plant cells [2]. Furthermore, we have shown previously that red beet microsomes and whole vacuoles release Ca^{2+} in response to sub-micromolar concentrations of cADPR [3].

The Ca^{2+}-mobilising properties of cADPR were first elucidated in sea urchin eggs [4]. Subsequently cADPR has been shown to effect Ca^{2+} release, via an $InsP_3$-insensitive pathway, from intracellular stores in a wide variety of animal cell types [5–7]. The exact mechanism by which cADPR releases Ca^{2+} is not known, and the protein(s) with which cADPR interacts to effect Ca^{2+} release have not yet been identified. The pharmacology of cADPR-induced Ca^{2+} release, however, bears many similarities with that of Ca^{2+} release via the ryanodine receptor, including modulation of release by ryanodine, activation by caffeine and inhibition by ruthenium red [6–10]. These observations, coupled with cADPR activation of ryanodine receptors reconstituted into planar lipid bilayers [11], suggest that cADPR may act as endogenous activator of at least one isoform of the ryanodine receptor in animal cells.

On the basis of cross-desensitisation studies, utilising cADPR, $InsP_3$ and ryanodine we have shown previously that cADPR acts to release Ca^{2+} via a mechanism distinct from that gated by $InsP_3$ in red beet [3]. We demonstrate here that cADPR-induced Ca^{2+} release from red beet is sensitive to modulation by ryanodine receptor agonists and antagonists in concentration ranges comparable with that observed in animal cells.

2. Materials and methods

2.1. Chemicals

$^{45}CaCl_2$ (spec. act. 2 Ci/mmol) was obtained from Amersham. High purity ryanodine was from Calbiochem. All other reagents were obtained from Sigma, except cADPR and $InsP_3$ which were generous gifts from Dr. Anthony Galione (University of Oxford, UK) and Dr. Robin Irvine (University of Cambridge, UK), respectively.

2.2. Preparation of red beet microsomes

Microsomes were isolated from red beet (*Beta vulgaris*), based on the protocol given in [12]. Storage root (330 g) of fresh, greenhouse grown red beet was homogenised at pH 8.0 in a Kenwood blender for approx. 30 s. 1 ml of homogenisation medium [12] was used per g tissue. The homogenate was filtered through two layers of muslin and centrifuged at $10000 \times g$ for 15 min. The supernatant was re-centrifuged at $80000 \times g$ for 30 min to give a crude microsomal pellet which was resuspended in a suspension medium at pH 8.0, as detailed in [12]. A further centrifugation step followed at $80000 \times g$ for 30 min. The final microsomal pellet was then resuspended in 5 ml of 400 mM glycerol, 5 mM Tris-Mes pH 7.4, 0.5 mM PMSF and 2 μg/ml leupeptin.

2.3. Ca^{2+} transport assay

Microsomes (30–50 μg) were resuspended in 1 ml of a Ca^{2+} uptake medium containing 400 mM glycerol, 5 mM Tris-Mes pH 7.4, 50 mM KCl, 3 mM $MgSO_4$, 3 mM Tris-ATP and 0.3 mM NaN_3.

Ca^{2+} uptake was initiated with the addition of 10 μM $CaCl_2$ spiked with 0.22 μCi $^{45}Ca^{2+}$ (original spec. act. 2.2 mCi/ml). Uptake was allowed to reach steady state levels for 19–20 min before the addition of 10 μM FCCP to abolish further uptake [13]. Further additions were made with rapid mixing. Aliquots of 50 μl were removed from the reaction medium and placed onto nitrocellulose filters (0.45 μm pore size: type WCN, Whatman) which had been prewetted with wash medium (400 mM glycerol, 0.2 mM $CaCl_2$ and 5 mM Tris-Mes pH 7.4). Filtration was then carried out using a Millipore filtration unit under vacuum. The membranes were rapidly washed once with 5 ml of ice-cold wash medium, placed in scintillation vials and radioactivity determined by liquid scintillation counting. Radioactivity remaining on the filters after the addition of the Ca^{2+} ionophore A23187 is defined as non-accumulated Ca^{2+} and was subtracted from all the data points. This correction never amounted to more than 25% of the overall maximum Ca^{2+} accumulation. Red beet vesicles do not show any significant 'leak' of $^{45}Ca^{2+}$ in the absence of effector over the time course of the experiment [13].

2.4. Protein determination

Protein concentration was determined using the Bio-Rad assay kit based on a modification of the dye binding method of Bradford [14]. Bovine serum albumin was used as a standard.

*Corresponding author. Fax: (44) (1904) 434317.
E-mail: srm3@unix.york.ac.uk

Abbreviations: cADPR, cyclic ADP-ribose; $InsP_3$, inositol 1,4,5-trisphosphate; Mes, 2-[*N*-morpholino]ethanesulphonic acid; PMSF, phenylmethylsulphonyl fluoride; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

PII S0014-5793(96)01000-9

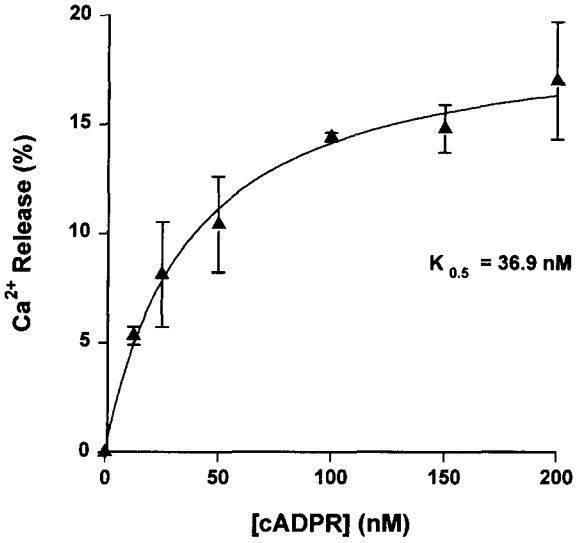

Fig. 1. Dose-response curve for cADPR-induced Ca^{2+} release. Red beet microsomes were allowed to accumulate Ca^{2+} for 19 min before the addition of varying concentrations of cADPR. Release is expressed as a percentage of the total A23187-sensitive Ca^{2+} pool (7.3 ± 0.3 nmol/mg). Data (mean ± SEM of three experiments) are fitted by the Michaelis-Menten equation using a non-linear least-squares fitting routine which yielded a $K_{0.5}$ of 36.9 ± 5.0 nM.

3. Results and discussion

Fig. 1 shows that cADPR induced Ca^{2+} release from red beet microsomes in a dose-dependent manner, with a $K_{0.5}$ (defined as the concentration of effector or inhibitor required for half-maximal activation or inhibition of Ca^{2+} release) of 36.9 ± 5.0 nM and a maximal release (R_{max}) of 19.3 ± 0.8% of the accumulated Ca^{2+}. The $K_{0.5}$ is in reasonable agreement with the value of 24 nM previously determined for activation of inward currents in red beet vacuoles using the patch-clamp technique [3] and also with values for Ca^{2+} release obtained from animal studies [9,15]. Ca^{2+} release from red beet microsomes was specific for the cyclic isomer since adenosine 5′-diphosphoribose at 1 μM was not effective at releasing Ca^{2+} (data not shown).

To distinguish between $InsP_3$- and cADPR-induced Ca^{2+} release from red beet we tested two Ca^{2+} channel antagonists previously characterised on animal systems (Table 1). Heparin (10 μM), a competitive inhibitor of $InsP_3$ binding in both animal and plant cells [16,17], was without effect on cADPR-induced Ca^{2+} release. Since previous studies [13] have shown that heparin at this concentration completely blocks $InsP_3$-induced Ca^{2+} release from red beet microsomes, this result strongly suggests that cADPR and $InsP_3$ are acting on different Ca^{2+} release pathways. This notion is substantiated by the finding that release of Ca^{2+} from red beet microsomes by saturating concentrations of cADPR and $InsP_3$ added sequentially occurs in an additive fashion [3].

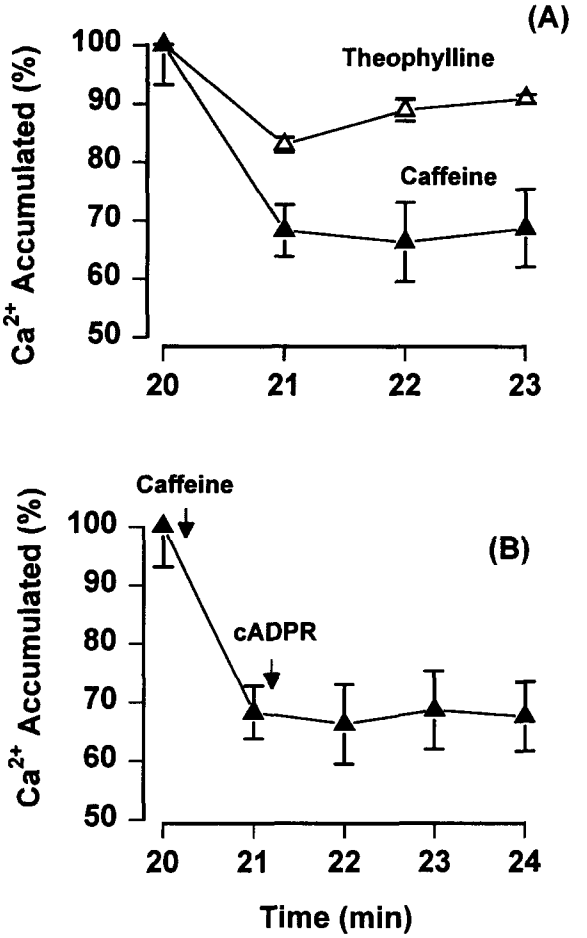

Fig. 2. Ca^{2+} release by caffeine precludes further Ca^{2+} release by cADPR. Red beet microsomes were allowed to accumulate Ca^{2+} for 19 min. (A) At t = 20 min, 5 mM caffeine (▲) or 5 mM theophylline (△) was added. 100% (A23187-sensitive) Ca^{2+} uptake was 9.8 ± 0.9 nmol/mg. Results are the means ± SEM of four (caffeine) or three (theophylline) experiments. (B) At t = 20 min Ca^{2+} release was initiated by the addition of caffeine (5 mM) then, 1 min later, cADPR (100 nM) was added. 100% (A23187-sensitive) Ca^{2+} accumulation = 11.6 ± 0.8 nmol/mg. Results are the means ± SEM of four experiments.

Procaine (1 mM) is an antagonist of cADPR-induced Ca^{2+} release in animal cells [6], and proved effective at inhibiting cADPR-induced release from red beet microsomes (Table 1), possibly suggesting a degree of conservation in the release mechanism.

Similarities in the mechanism of cADPR-induced Ca^{2+} release between animals and red beet were further confirmed by the action of caffeine. Caffeine acts as an activator of ryano-

Table 1
Inhibitor sensitivity of cADPR-induced Ca^{2+} release

Inhibitor	Ca^{2+} release by 100 nM cADPR (nmol/mg)	Inhibition of cADPR-induced Ca^{2+} release (%)
Control (no inhibitor)	1.07 ± 0.21	–
10 μM heparin	1.01 ± 0.06	0.9
1 mM procaine	0.10 ± 0.077	5.2

cADPR (100 nM) was added to Ca^{2+}-loaded red beet microsomes in either the absence (control) or presence of low M_r heparin (10 μM, based on M_r = 5000) and procaine (1 mM). Potential inhibitors were added 1 min prior to the addition of cADPR. Results are the means ± SEM of four experiments except for the control (n = 5).

dine receptors and has been used in conjunction with cADPR in cross-desensitisation studies to investigate whether cADPR acts as a physiological regulator of the ryanodine receptor [5]. When caffeine (5 mM) was added to Ca^{2+}-loaded red beet microsomes it released 32% of the accumulated Ca^{2+} (Fig. 2A). However, the low potency of caffeine, and hence the requirement to conduct caffeine assays at mM concentrations, coupled with its limited solubility in aqueous solutions, made it necessary to add 100 μl of a stock solution of 50 mM caffeine to the assay. This addition will lead to a dilution of the assay volume, causing a 10% decrease in the concentration of $^{45}Ca^{2+}$. In order to correct for this dilution effect, the closely related methylxanthine, theophylline, was used as a control [18] and added to the assay at an identical concentration and volume. Addition of theophylline (5 mM) resulted in a 10% reduction in accumulated Ca^{2+} over a similar time scale, thus demonstrating that a major component of the caffeine-induced Ca^{2+} release is specific.

Significantly, following release by caffeine, subsequent addition of a saturating concentration of cADPR (100 nM) failed to release any further Ca^{2+} (Fig. 2B). This result is similar to that observed in sea urchin egg homogenates [6] and suggests that caffeine may act through the same release pathway as cADPR in red beet.

Ryanodine is a plant alkaloid which acts as a modulator of the ryanodine receptor in animal cells [19,20]. Two classes of binding site for [^{3}H]ryanodine have been identified – a high affinity site with an apparent K_d between 2 and 200 nM (depending upon experimental conditions) and a low affinity site(s) with an apparent K_d of greater than 1 μM [19]. The complexity of ryanodine binding explains why, depending upon the receptor isoform and the concentration of ryanodine used, ryanodine can either activate or inhibit Ca^{2+} release in animal cells.

We have shown previously that ryanodine, at a relatively high concentration of 100 μM, is capable of releasing Ca^{2+}

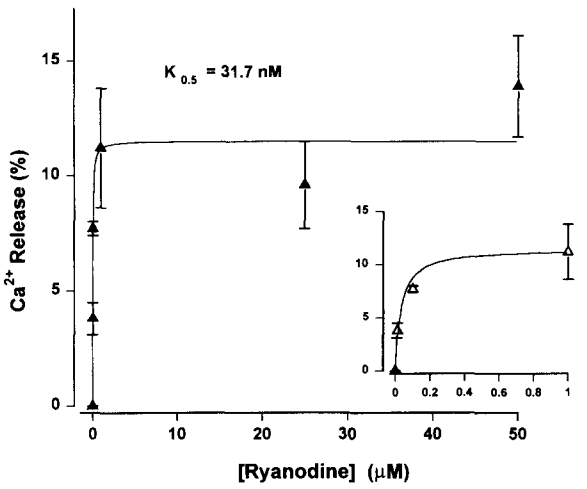

Fig. 3. Dose-response curve for ryanodine-induced Ca^{2+} release. Red beet microsomes were allowed to accumulate Ca^{2+} for 20 min before the addition of varying concentrations of ryanodine. Release is expressed as a percentage of the total A23187-sensitive Ca^{2+} pool (10.7 ± 0.8 nmol/mg). Data (mean ± SEM of four experiments) are fitted to the Michaelis-Menten equation using a non-linear least-squares fitting routine which yielded a $K_{0.5}$ of 31.7 ± 19.0 nM. (Inset) Expanded abscissa showing ryanodine-dependent Ca^{2+} release at sub-micromolar concentrations.

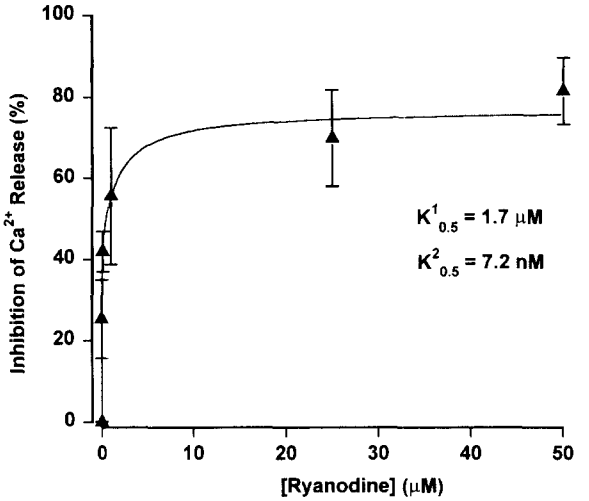

Fig. 4. Dose-response curve for ryanodine inhibition of cADPR-induced Ca^{2+} release. Red beet microsomes were allowed to accumulate Ca^{2+} for 20 min before the addition of varying concentrations of ryanodine followed, 3 min later, by the addition of cADPR (100 nM). 100% cADPR-elicited Ca^{2+} release = 1.1 ± 0.2 nmol Ca^{2+}/mg. Data are the mean ± SEM of four experiments except for ryanodine concentrations 0.1 μM and 1 μM ($n = 3$). Solid line shows a non-linear least-squares fit to the sum of two Michaelis-Menten equations, which yielded a $K^1_{0.5}$ of 1.7 ± 1.3 μM and a $K^2_{0.5}$ of 7.2 ± 2.4 nM.

from red beet microsomes, and that pre-release by ryanodine precludes further Ca^{2+} release by a saturating concentration of cADPR [3]. In order to investigate the action of ryanodine in more detail we tested varying concentrations of ryanodine for the ability to release Ca^{2+} from red beet microsomes and the ability to inhibit subsequent Ca^{2+} release by a saturating dose of cADPR. Ryanodine-induced Ca^{2+} release from red beet microsomes was dose-dependent and the data conformed to a first order Michaelis-Menten relationship with derived values for $K_{0.5}$ and R_{max} of 31.7 ± 19.0 nM and 11.5 ± 1.0%, respectively (Fig. 3). The value of R_{max} obtained in this study is consistent with reports from the animal literature: in hepatic microsomes ryanodine at concentrations of 50 μM or above released 20% of accumulated Ca^{2+} [21]. Similarly, in rabbit brain microsomes 100 μM ryanodine caused release of 20% of sequestered Ca^{2+} [22]. Dose-dependent release by ryanodine has been reported in sea urchin eggs, although in this system a minimal concentration of 2 μM ryanodine was required to elicit significant Ca^{2+} release [8]. Dose-dependent release by ryanodine has also been reported in actively loaded skeletal SR vesicles, where Ca^{2+}-induced Ca^{2+} release was significantly enhanced by ryanodine in the nanomolar range [23].

Significantly, ryanodine also inhibited Ca^{2+} release by a subsequent addition of a saturating concentration of cADPR in a dose-dependent manner (Fig. 4). However, the kinetics of ryanodine inhibition suggested that ryanodine acts at two binding sites: one with high affinity and a $K_{0.5}$ = 7.2 ± 2.4 nM, and one with low affinity and a $K_{0.5}$ = 1.7 ± 1.4 μM. These values are very similar to the K_d for the low and high affinity [^{3}H]ryanodine binding sites on the animal ryanodine receptor [20].

At present there is no molecular evidence for the existence of a ryanodine-like Ca^{2+} release channel existing in plants. However, the finding that red beet microsomes are sensitive to cADPR, ryanodine and caffeine argues strongly for the presence of such a Ca^{2+} release pathway in plant cells. More-

over, the respective potencies of these ligands are within the ranges reported for animal systems thus arguing for a evolutionary conservation of the Ca^2 release mechanism.

Acknowledgements: We thank the B.B.S.R.C. for the award of a studentship to S.R.M.

References

[1] Bush, D.S. (1995) Annu. Rev. Plant Physiol. 46, 95–122.
[2] Coté, G.G. and Crain, R.C. (1994) BioEssays 16, 39–46.
[3] Allen, G.J., Muir, S.R. and Sanders, D. (1995) Science 268, 735–737.
[4] Clapper, D.L., Walseth, T.F., Dargie, P.J. and Lee, H.C. (1987) J. Biol. Chem. 262, 9561–9568.
[5] Galione, A. (1994) Mol. Cell. Endocrinol. 98, 125–131.
[6] Galione, A., Lee, H.C. and Busa, W.B. (1991) Science 253, 1143–1146.
[7] Gromada, J., Jørgensen, T.D. and Dissing, S. (1995) FEBS Lett. 360, 303–306.
[8] Buck, W.R., Hoffman, E.E., Rakaw, T.L. and Shen, S.S. (1994) Dev. Biol. 163, 1–10.
[9] Lee, H.C. (1993) J. Biol. Chem. 268, 293–299.
[10] Palade, P., Dettbarn, C., Brunder, D., Stein, P. and Hals, G. (1989) J. Bioenerg. Biomembr. 21, 295–320.
[11] Meszaros, L.G., Bak, J. and Chu, A. (1993) Nature 364, 76–79.
[12] Rea, P.A. and Poole, R.J. (1985) Plant Physiol. 77, 46–52.
[13] Brosnan, J.M. and Sanders, D. (1990) FEBS Lett. 260, 70–72.
[14] Bradford, M. (1976) Anal. Biochem. 72, 248–256.
[15] Takasawa, S., Nata, K., Yonekura, H. and Okamoto, H. (1993) Science 259, 370–373.
[16] Worley, P.F., Baraban, J.M., Supattapone, S., Wilson, V.S. and Synder, S.H. (1987) J. Biol. Chem. 262, 12132–12136.
[17] Brosnan, J.M. and Sanders, D. (1993) Plant Cell 5, 931–940.
[18] McNulty, T.J. and Taylor, C.W. (1993) Biochem. J. 291,799–801.
[19] Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q.-Y. and Meissner, G. (1988) Nature 331, 315–319.
[20] Zimanyi, I. and Pessah, I.N. (1994) Handbook of Membrane Channels: Molecular and Cellular Physiology, Academic Press, London.
[21] Lilly, L.B. and Gollan, J.L. (1995). Am. J. Physiol. Gastroint. Liver Physiol. 31, G1017–G1024.
[22] White, A.M., Watson, S.P. and Galione, A. (1993) FEBS Lett. 318, 259–261.
[23] Palade, P. (1987) J. Biol. Chem. 262, 6135–6141.