ELSEVIER

European Journal of Pharmacology 354 (1998) 245–251

ejp

2-Hydroxycarbazole induces Ca^{2+} release from sarcoplasmic reticulum by activating the ryanodine receptor

Stephen C. Tovey, Clare L. Longland, Mokdad Mezna, Francesco Michelangeli *

School of Biochemistry, University of Birmingham, Edgbaston, Birmingham, UK B15 2TT

Received 26 February 1998; revised 19 May 1998; accepted 9 June 1998

Abstract

2-Hydroxycarbazole was shown to induce Ca^{2+} release from skeletal muscle and cardiac muscle sarcoplasmic reticulum at concentrations between 100–500 μM. This release was blocked by both 1 mM tetracaine and 30 μM ruthenium red which inhibit the ryanodine receptor or by pre-treatment with 10 mM caffeine which depletes the ryanodine receptor-containing Ca^{2+} stores. This, in addition to the fact that 2-hydroxycarbazole has little effect on Ca^{2+} ATPase activity, indicates that it activates Ca^{2+} release through the ryanodine receptor. The apparent EC_{50} value for release from both skeletal muscle and cardiac muscle sarcoplasmic reticulum was ≈ 200 μM and maximal release occurred at 400–500 μM, making it ≈ 20 times more potent than caffeine. The dose-dependency in the extent of Ca^{2+} release induced by 2-hydroxycarbazole was also apparently highly cooperative for both preparations. That 2-hydroxycarbazole was able to mobilize Ca^{2+} from non-muscle cell microsomes and in intact TM4 cells (which contain ryanodine receptors), makes this compound a more potent and commercially available alternative to caffeine in studying the role of this intracellular Ca^{2+} channel in a variety of systems. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: 2-Hydroxycarbazole; Ca^{2+} release; Ryanodine receptor

1. Introduction

In recent years, it has been demonstrated that a number of pharmacological agents can induce Ca^{2+} release from skeletal muscle and cardiac muscle sarcoplasmic reticulum by the direct activation of ryanodine receptors (Palade et al., 1989). However, many have proved to have additional non-specific effects which limit their application or as in the case of cyclic ADP-ribose which is isoform specific (Meszaros et al., 1993). Probably the most widely used pharmacological agent that stimulates Ca^{2+} release from the ryanodine receptor is caffeine (1,3,7-trimethyl xanthine). It has been demonstrated that mM concentrations of caffeine can stimulate Ca^{2+} release from skeletal muscle and cardiac muscle sarcoplasmic reticulum with maximal Ca^{2+} release occurring at a caffeine concentration of approximately 10–20 mM (Endo, 1977; Palade et al., 1989; Rousseau et al., 1988). In addition, caffeine can mobilize Ca^{2+} from non-muscle cells such as permeabilized and intact adrenal chromaffin cells (Cheek et al., 1990, 1991) Although caffeine is the most widely used and best characterized of the agents that mobilize Ca^{2+} from the ryanodine receptor, it has many disadvantages that makes its utilization as a pharmacological tool limited. The low potency of caffeine means that it cannot be used effectively in binding experiments and it is also relatively insoluble at the high concentrations required to evoke Ca^{2+} release. It has been demonstrated that mM concentrations of caffeine also inhibit inositol 1,4,5-trisphosphate ($InsP_3$)-induced Ca^{2+} release from both *Xenopus* oocytes (Parker and Ivorra, 1991) and rat cerebellar microsomes (Brown et al., 1992) and it also inhibits phosphodiesterase activity. Various other methyl xanthines have been shown to mobilize Ca^{2+} from the ryanodine receptor including 1,7-dimethyl xanthine and 3,7-dimethyl xanthine, but these have relatively similar potencies to caffeine (Rousseau et al., 1988).

More recently, bromoeudistomin D and 9-methyl-7-bromoeudistomin D, isolated from the caribbean tunicate *Eudistoma olivaceum*, were both found to be highly potent at mobilizing Ca^{2+} from the heavy fraction of skeletal muscle sarcoplasmic reticulum (Nakamura et al., 1986). Structurally these compounds consist of a brominated β-

* Corresponding author. Tel.: +44-121-414-5398; Fax: +44-121-414-3982; e-mail: f.michelangeli@bham.ac.uk

0014-2999/98/$19.00 © 1998 Elsevier Science B.V. All rights reserved.

carboline skeleton and both release Ca^{2+} at μM concentrations with a potency ≈300 times higher than caffeine (Kobiyashi et al., 1989, Nakamura et al., 1986, Seino et al., 1991).

The problems involved in using caffeine to mobilize Ca^{2+} through the ryanodine receptor, as highlighted above, make it unattractive as a tool with which to study ryanodine receptor function. Bromoeudistomin D and analogues of eudistomin are not commercially available and as such, this study has looked for compounds that are structurally similar, commercially available and inexpensive. Here we show that the commercially available compound, 2-hydroxycarbazole, is capable of mobilizing Ca^{2+} from both skeletal muscle heavy sarcoplasmic reticulum, cardiac muscle sarcoplasmic reticulum and cerebellar microsomes specifically through the ryanodine receptor with ≈20 times the potency of caffeine.

2. Materials and methods

2-Hydroxycarbazole was purchased from Aldrich Chemical (catalogue no. 21,349-7). Concentrated stock solutions were prepared in dimethylsulphoxide and kept in the dark. Dimethylsulphoxide alone at the volumes added as the vehicle (<2.5%) did not affect Ca^{2+} release from sarcoplasmic reticulum, microsomes or cells and had little or no effect on Ca^{2+} ATPase activity or [^{3}H]ryanodine binding to sarcoplasmic reticulum.

Rabbit skeletal muscle heavy sarcoplasmic reticulum was prepared as described by Siato et al. (1984). Rat cardiac sarcoplasmic reticulum and cerebellar microsomes were prepared as described by Michelangeli (1990) and Mezna and Michelangeli (1997). The tissues were homogenized using either a teflon Potter–Elvehjem homogenizer or Polytron (for heart tissue) in 10 vols of 0.32 M sucrose, 5 mM Hepes, pH 7.2 in the presence of protease inhibitors (0.1 mM phenylmethylsulphonyl fluoride, 10 μM leupeptin, and 0.1 mM benzamidine) and centrifuged at 500 $\times g$ for 10 min. The supernatant was then centrifuged for 10 min at 10 000 $\times g$ and the resultant supernatant centifuged for a further 1 h at 100 000 $\times g$. The microsomal pellet was resuspended in Hepes/sucrose buffer, frozen in liquid nitrogen and stored at −70°C until use.

Ca^{2+} uptake and release were measured using fluo-3 as described by Brown et al. (1992). Briefly, sarcoplasmic reticulum or microsomes were suspended in a buffer containing 60 mM KCl, 40 mM potassium phosphate, 3.5 mM potassium pyrophosphate, 250 nM fluo-3, 10 μg/ml creatine kinase and 10 mM phosphocreatine, pH 7.2. Heavy sarcoplasmic reticulum (50 μg), cardiac sarcoplasmic reticulum (0.5 mg) or cerebellar microsomes (0.5 mg) was added to 2 ml of this buffer in a stirred cuvette and Ca^{2+} uptake was initiated by the addition of 1.5 mM Mg–ATP. The change in fluorescence was monitored by measuring the emission at 526 nm, while exciting the sample at 506 nm. Once sufficient Ca^{2+} had accumulated within the vesicles, 2-hydroxycarbazole was added. In experiments where cerebellar microsomes were used, further Ca^{2+} uptake was inhibited with 0.5 mM orthovanadate prior to adding either 2-hydroxycarbazole or inositol 1,4,5-trisphosphate ($InsP_3$). Total accumulated Ca^{2+} within the sarcoplasmic reticulum or microsomes was measured by the addition of 12.5 μg/ml A23187. The fluorescence intensity was related to [Ca^{2+}] using the equation described by Michelangeli (1990) (the K_d for Ca^{2+} binding fluo-3 was determined to be 900 nM at pH 7.2). The Ca^{2+} ATPase activity was measured using the coupled enzyme assay described by Michelangeli and Munkonge (1990).

TM4 cells (a cell line derived from testicular Sertoli cells) were grown in a medium containing 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture, supplemented with 5% (v/v) foetal calf serum, 2.5% (v/v) horse serum, 100 U/ml penicillin, 100 U/ml streptomycin and 8 mM L-glutamine. The cells were removed from plates by treatment with trypsin/EDTA for 5 min at 37°C. Intracellular Ca^{2+} measurements were monitored by loading the cells with fluo-3AM. The cells were resuspended in Hank's balanced salt solution containing 1% bovine serum albumin, to which was added 5 μM fluo-3AM and incubated for 30 min at 37°C. The cells were then washed in fresh solution and resuspended to a concentration of 1.5 million cells/ml. Cells amounting to 2 ml were then placed into a cuvette and the fluorescence monitored at 25°C (exciting at 505 nm and measuring the emission at 526 nm). The fluorescence changes were related to free Ca^{2+} concentration as described by Minta et al. (1989). Ca^{2+} measurements were carried out in the absence of extracellular Ca^{2+} by adding 30 mM EGTA, so that any change in intracellular Ca^{2+} levels could only be attributed to mobilization of intracellular Ca^{2+} stores.

The extent of [^{3}H]ryanodine binding to heavy sarcoplasmic reticulum membranes was measured as described by Lai et al. (1988). Approximately 1 mg of membranes were suspended in 1 ml of buffer containing 1 M NaCl, 10 mM AMP, 20 mM 1,4-piperazinediethanesulphonic acid (Pipes), at pH 7.1 supplemented with 150 μM $CaCl_2$ and 100 μM EGTA to give a free Ca^{2+} concentration of approximately 50 μM. The buffer was doped with [^{3}H]ryanodine and specific binding was measured at a ryanodine concentration of 20 nM (specific activity 250 Ci/mol). The non-specific binding was measured in the presence of 100 μM excess non-radioactive ryanodine. After adding membranes to the assay mixture each sample was incubated for 3 h at 37°C. Bound [^{3}H]ryanodine was separated from free by rapid filtration onto Whatman GF/C filters using a vacuum manifold filtration system, each filter was then rapidly washed with 3 × 5 ml ice-cold buffer. The filter was air dried for 30 min, added to 5 ml Ultima flow scintillant (ammonium formate) and the radioactivity of each sample determined by scintillation

spectrometry, counting for 5 min per sample. Specific [^{3}H]ryanodine bound was expressed as pmol [^{3}H]ryanodine bound per mg of membrane protein. The effects of differing 2-hydroxycarbazole concentrations on specific [^{3}H]ryanodine binding was investigated by pre-incubating membranes with the compound for 5 min prior to binding.

3. Results

The characterization of 2-hydroxycarbazole-induced Ca^{2+} release from skeletal muscle heavy sarcoplasmic reticulum was carried out using the Ca^{2+} indicating dye fluo-3. Ca^{2+} uptake was initiated by the addition of 1.5 mM Mg–ATP. The rate of Ca^{2+} uptake into heavy sarcoplasmic reticulum was calculated to be 12.1 ± 0.6 nmol of Ca^{2+}/min/mg of protein at 25°C. Following the completion of Ca^{2+} uptake increasing concentrations of 2-hydroxycarbazole were added, in order to assess its effect upon Ca^{2+} release. Fig. 1A shows that Ca^{2+} release from heavy sarcoplasmic reticulum increases with increasing concentrations of 2-hydroxycarbazole. Fig. 1B also shows Ca^{2+} release from cardiac muscle sarcoplasmic reticulum increases with increasing concentrations of 2-hydroxycarbazole, reaching a maximum again at a concentration of approximately 500 μM.

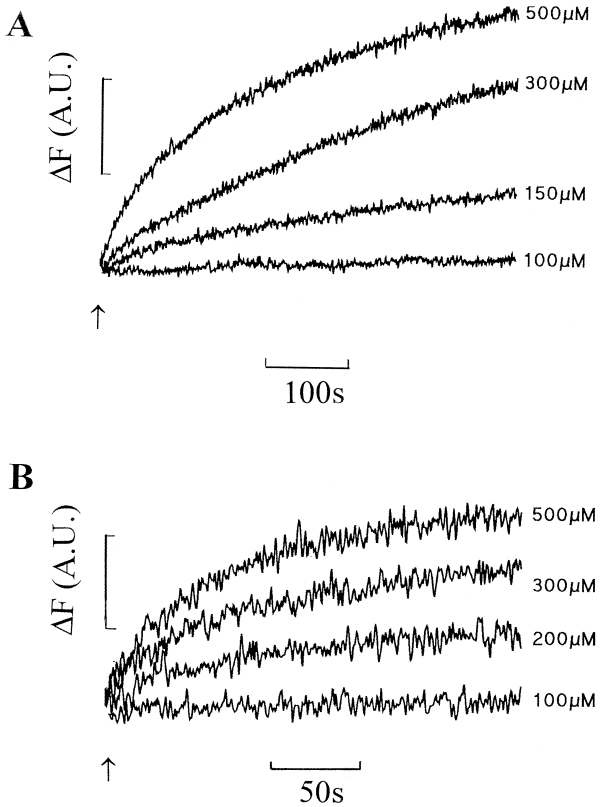

Fig. 1. The effects of 2-hydroxycarbazole on Ca^{2+} release in skeletal muscle and cardiac muscle sarcoplasmic reticulum. (A) The effects of 2-hydroxycarbazole on Ca^{2+} release from skeletal muscle heavy sarcoplasmic reticulum (100–500 μM). The traces are given in changes in fluo-3 fluorescence intensities, with maximal release occurring at 500 μM and causing 10.2% of the accumulated Ca^{2+} to be released. Each trace is representative of three or more determinations. (B) The effects of 2-hydroxycarbazole on Ca^{2+} release from cardiac muscle sarcoplasmic reticulum (100–500 μM). Ca^{2+} release induced by 500 μM 2-hydroxycarbazole was calculated to be 6.9% of the accumulated Ca^{2+}. Each trace is representative of three or more determinations. The arrow marks the addition of 2-hydroxycarbazole.

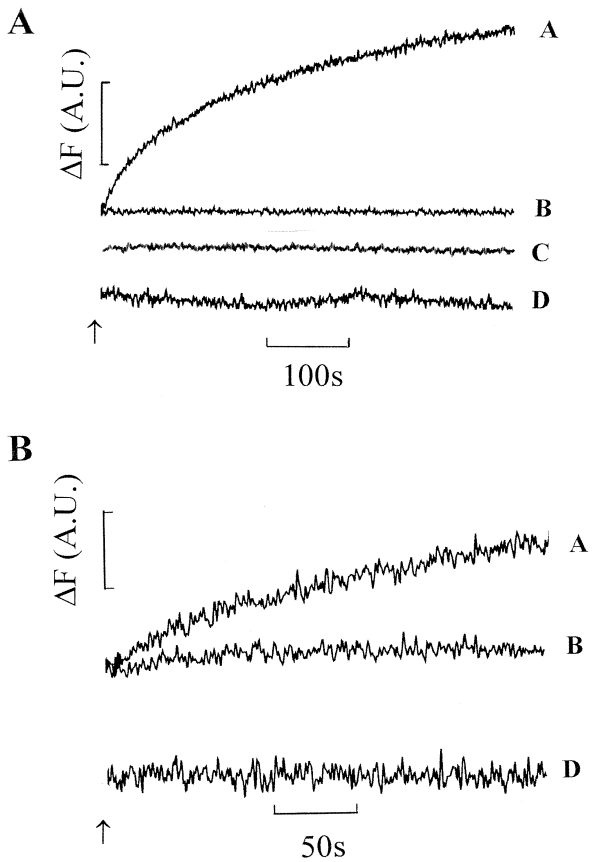

Fig. 2. Inhibition of 2-hydroxycarbazole-induced Ca^{2+} release by tetracaine and ruthenium red or depletion of the caffeine-sensitive Ca^{2+} stores. (A) The traces represent the effects of 2-hydroxycarbazole on Ca^{2+} release in skeletal muscle heavy sarcoplasmic reticulum. (B) The traces represent the effects of 2-hydroxycarbazole on Ca^{2+} release from cardiac muscle sarcoplasmic reticulum. The trace labelled A shows the effects of 500 μM 2-hydroxycarbazole on Ca^{2+} release. Traces labelled B are the effects of 2-hydroxycarbazole on Ca^{2+} release from skeletal and cardiac muscle sarcoplasmic reticulum after pre-treatment with 1 mM tetracaine. The traces labelled D are the effects of 2-hydroxycarbazole on Ca^{2+} release from sarcoplasmic reticulum in the presence of 30 μM ruthenium red. The trace labelled C is Ca^{2+} release by 2-hydroxycarbazole following the addition of 10 mM caffeine. These traces have been displaced downwards to clarify that ruthenium red completely blocks 2-hydroxycarbazole-induced Ca^{2+} release. All the traces are representative of at least three separate determinations. The arrow marks the addition of 2-hydroxycarbazole.

Fig. 2A, traces B and D show that Ca^{2+} release by 2-hydroxycarbazole from skeletal muscle sarcoplasmic reticulum could be blocked by the addition of either 1 mM tetracaine or 30 μM ruthenium red. The local anaesthetic tetracaine inhibits Ca^{2+} release from both $InsP_3Rs$ and ryanodine receptors (Palade et al., 1989), while ruthenium red is a specific inhibitor of Ca^{2+} release from ryanodine

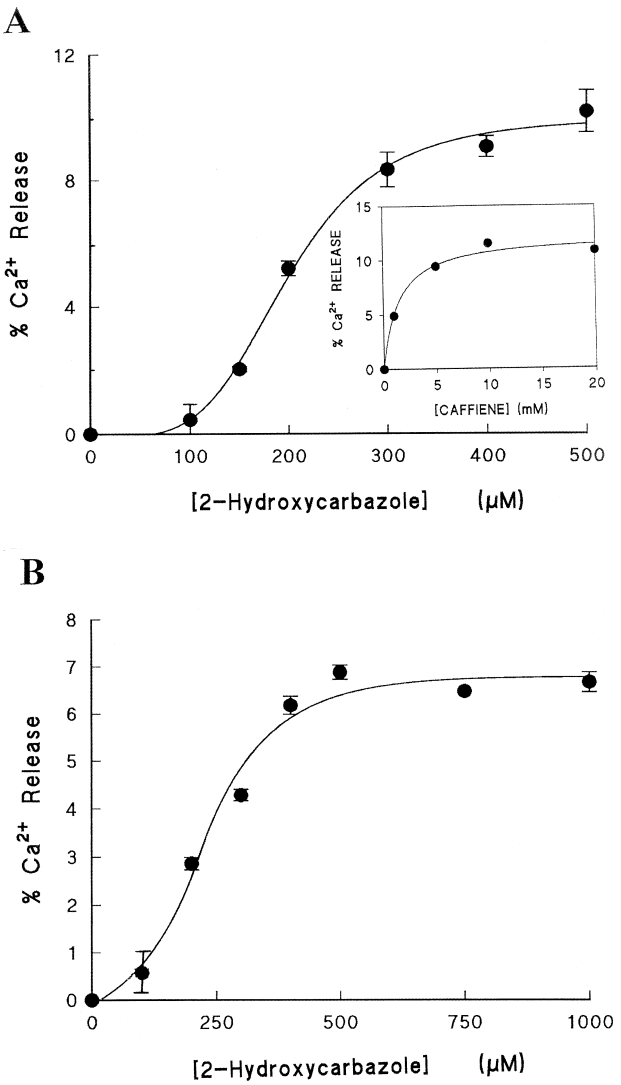

Fig. 3. Dose-dependency of Ca^{2+} release induced by 2-hydroxycarbazole. (A) shows the effects of 2-hydroxycarbazole on the extent of release in skeletal muscle heavy sarcoplasmic reticulum. The data is presented as the percentage of Ca^{2+} release compared to that released by A23187. The inset shows the dose-dependency of caffeine on Ca^{2+} release under similar conditions. (B) shows the effect of 2-hydroxycarbazole on the extent of Ca^{2+} release from cardiac sarcoplasmic reticulum. Each data point is the mean ± S.E. of three or more determinations.

receptors (Meissner et al., 1986). In addition, no release by 2-hydoxycarbazole was observed after depleting the ryanodine receptor containing Ca^{2+} stores with 10 mM caffeine (Fig. 2A, trace C). This suggests that 2-hydroxycarbazole-induced Ca^{2+} release from skeletal muscle heavy sarcoplasmic reticulum occurs via activation of the ryanodine receptor. Fig. 2B shows that 2-hydroxycarbazole (500 μM) induces Ca^{2+} release from cardiac muscle sarco-plasmic reticulum which could also be blocked by the addition of 1 mM tetracaine or 30 μM ruthenium red (Fig. 2B, traces B and D), similar to skeletal muscle sarcoplasmic reticulum.

Fig. 3A shows the dose-dependent mobilization of Ca^{2+} from skeletal muscle sarcoplasmic reticulum induced by 2-hydroxycarbazole. The amount of Ca^{2+} released with increasing 2-hydroxycarbazole concentration was expressed as a percentage of that released by the addition of 12.5 μg/ml Ca^{2+} ionophore A23187. Maximal Ca^{2+} release occurred at a 2-hydroxycarbazole concentration of 400–500 μM and constituted 10.2 ± 0.7% release compared to the Ca^{2+} released by the ionophore. The EC_{50} for 2-hydroxycarbazole-induced Ca^{2+} release was found to be 200 ± 9 μM. The release process was found to be highly cooperative with a Hill coefficient of 3.9 ± 0.4. However, it must be noted that since these experiments were undertaken when the Ca^{2+} ATPases were active, these parameters should be considered as apparent values. At concentrations of 2-hydroxycarbazole above 500 μM, a continual slow steady rise in Ca^{2+} was observed after the initial phase of Ca^{2+} mobilization. This rise in Ca^{2+} was probably due to passive leakage since the Ca^{2+} ATPase was partially inhibited at high 2-hydroxycarbazole concentrations (see Table 1). The inset to Fig. 3A shows that under identical conditions caffeine is far less potent at releasing Ca^{2+} from skeletal muscle sarcoplasmic reticulum. Caffeine of 10 mM was required to release maximal Ca^{2+}, thus making 2-hydroxycarbazole ≈ 20 times more potent than caffeine at releasing Ca^{2+}. Fig. 3B shows the dose-dependent mobilization of Ca^{2+} from cardiac muscle sarcoplasmic reticulum induced by 2-hydroxycarbazole. Again, maximal Ca^{2+} release occurred at a concentration of approximately 400–500 μM causing 6.9 ± 0.2% Ca^{2+} release compared to ionophore and with an apparent EC_{50} value of 214 ± 24 μM, similar to skeletal muscle sarcoplasmic reticulum, as was the apparent Hill coefficient (3.0 ± 0.5). High concentrations of 2-hydroxycarbazole, however, did not appear to cause non-specific Ca^{2+} leakage from cardiac muscle sarcoplasmic reticulum, suggesting that high concentrations of 2-hydroxycarbazole may not be affecting the heart isoform of the Ca^{2+} ATPase.

In addition to Ca^{2+} release induced from skeletal muscle sarcoplasmic reticulum and cardiac muscle sarcoplasmic reticulum, 2-hydroxycarbazole was also shown to

Table 1
The effects of 2-hydroxycarbazole on the Ca^{2+}-ATPase activity and [^{3}H]ryanodine binding to skeletal muscle sarcoplasmic reticulum

[2-Hydroxycarbazole] (μM)	Ca^{2+}-ATPase activity (IU/mg)	[^{3}H]ryanodine bound (pmol/mg)
0.00	6.8 ± 0.5	6.0 ± 0.7
150	N.D.	6.1 ± 0.7
200	6.8 ± 0.3	N.D.
300	6.5 ± 0.4	6.0 ± 0.3
400	5.8 ± 0.5	N.D.
500	5.4 ± 0.4	5.0 ± 0.4

N.D.: not determined.
The values are the mean ± S.E. of three to five determinations.

mobilize Ca^{2+} from rat cerebellar microsomes. Fig. 4B shows that 300 μM 2-hydroxycarbazole mobilized 2.5 ± 0.4% of the Ca^{2+} released by the ionophore A23187, the subsequent addition of 20 μM $InsP_3$ resulted in the further mobilization of 11.4 ± 1.4% of the stored Ca^{2+} in cerebellar microsomes. Conversely, the addition of 20 μM $InsP_3$ mobilized 14.2 ± 0.1% of the stored Ca^{2+} from rat cerebellar microsomes, but the subsequent addition of 300 μM 2-hydroxycarbazole failed to mobilize any further Ca^{2+}. This suggests that in cerebellar microsomes 2-hydroxycarbazole mobilized Ca^{2+} comes from a store that was also $InsP_3$ sensitive. However, after depletion of the $InsP_3$-sensitive Ca^{2+} stores, no further Ca^{2+} was mobilized by 2-hydroxycarbazole confirming the observations that in cerebellar Purkinje cells (which are cells that contain most of the intracellular Ca^{2+} channels) there are regions rich only in $InsP_3$ receptors and regions rich in ryanodine receptors and $InsP_3$ receptors, but little or no regions rich only in ryanodine receptors (Walton et al., 1991).

2-Hydroxycarbazole did not significantly affect the specific [^{3}H]ryanodine binding to its receptor. Specific binding was found to constitute 6.00 ± 0.7 pmol/mg of membrane protein in the absence of 2-hydroxycarbazole (control). [^{3}H]ryanodine binding only appeared to be affected at high concentrations of 2-hydroxycarbazole (> 500 μM),

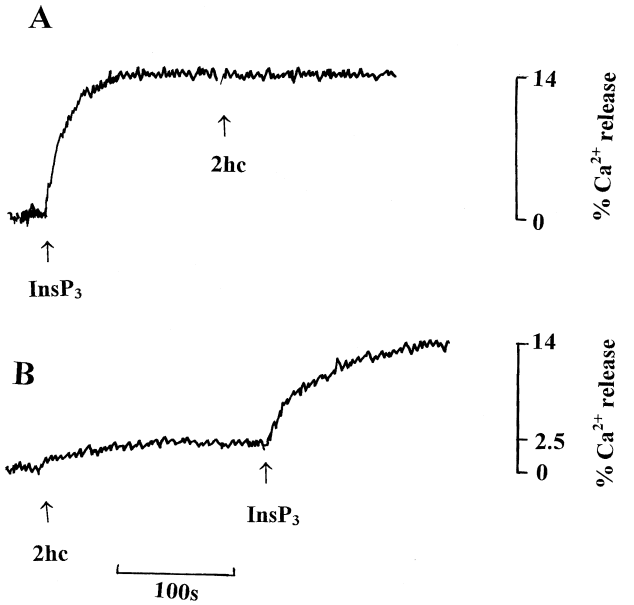

Fig. 4. The effects of 2-hydroxycarbazole on Ca^{2+} release from cerebellar microsomes. Trace A shows the effects of $InsP_3$ (20 μM) induced Ca^{2+} release from cerebellar microsomes added at the arrow labelled $InsP_3$. This caused 14.2% release of the accumulated Ca^{2+}. Subsequent addition of 300 μM 2-hydroxycarbazole (arrow labelled 2 hc) induced no further Ca^{2+} release. Trace B shows that if 300 μM 2-hydroxycarbazole is added prior to depletion of the $InsP_3$-sensitive Ca^{2+} stores (arrow labelled 2 hc) it is able to mobilize only a small amount of Ca^{2+} (2.5%). Subsequent addition of 20 μM $InsP_3$ (arrow labelled $InsP_3$) induced further Ca^{2+} release (11.4%).

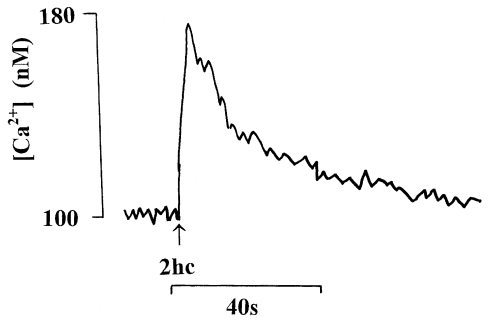

Fig. 5. 2-Hydroxycarbazole mobilizes intracellular Ca^{2+} from intact TM4 cells. The trace shows the effect of addition of 2-hydroxycarbazole (100 μM) on intact TM4 cells loaded with fluo-3AM (1.5 million cells/ml). The buffer contains very low external Ca^{2+} indicating that the Ca^{2+} rise must be due to mobilization from intracellular Ca^{2+} stores.

where a small decrease in binding was observed (Table 1). Therefore, the site of action for 2-hydroxycarbazole appears to be distinct from that of the ryanodine binding site. Table 1 also shows that 2-hydroxycarbazole had only a small effect on the activity of the skeletal muscle Ca^{2+} ATPase and then only at high concentrations. This fact and the fact that Ca^{2+} release induced by 2-hydroxycarbazole is not observed in the presence of ruthenium red or tetracaine once the caffeine sensitive Ca^{2+} stores have been depleted, argues against this release being due to non-specific leakage.

Fig. 5 shows that in the presence of very low external Ca^{2+}, 2-hydroxycarbazole can also mobilize Ca^{2+} from intracellular stores of intact TM4 cells (which are known to contain $InsP_3$ receptors (Tovey et al., 1997) and more recently by the use of reverse transcriptase polymerase chain reaction, also ryanodine receptors (Tovey and Michelangeli, unpublished observation). This, therefore suggests that 2-hydroxycarbazole is able to permeate through the plasma membrane and release Ca^{2+} from ryanodine receptor-containing stores.

4. Discussion

The contraction of skeletal and cardiac muscle is mediated by an increase in the concentration of cytosolic Ca^{2+} (Fleischer and Inui, 1989). The increase in Ca^{2+} responsible for muscle contraction occurs as a direct result of the activation of ryanodine receptor Ca^{2+} channels present in the sarcoplasmic reticulum membrane of muscle cells (Fleischer and Inui, 1989). The ryanodine receptor is also thought to play a key role in the generation and propagation of Ca^{2+} waves in non-muscle cells (Cheek et al., 1990, 1991), making the study of Ca^{2+} release from this channel important in understanding signalling processes in both muscle and non-muscle tissues.

The demonstration that bromoeudistomin D and a variety of its analogues are potent Ca^{2+} mobilizing agents

from skeletal muscle sarcoplasmic reticulum (Nakamura et al., 1986), lead us to look for compounds exhibiting structural similarities to bromoeudistomin D. The resulting search for a commercially available compound similar to bromoeudistomin D led to the discovery of 2-hydroxycarbazole. The structure of 2-hydroxycarbazole differs from bromoeudistomin D in that it is based on a carbazole ring rather than a β-carboline ring, although both are based on a skeleton of three aromatic rings. It has, however, been demonstrated that a non-commercially available halogenated carbazole derivative, 4,6-dibromo-3-hydroxycarbazole (100 μM) inhibits Ca^{2+} mobilization by both bromoeudistomin D (10 mM) and caffeine (1 mM), suggesting that the carbazole structure can be substituted for the β-carboline structure and that the mobilization of Ca^{2+} from skeletal muscle sarcoplasmic reticulum by this family of pharmacological agents is highly stereospecific in nature (Takahashi et al., 1995b).

The mobilization of Ca^{2+} from both skeletal muscle sarco-plasmic reticulum and cardiac muscle sarcoplasmic reticulum occurred over a 2-hydroxycarbazole concentration range of 0–500 μM. In both cases, 2-hydroxycarbazole induced Ca^{2+} release appeared to be biphasic in nature, consisting of a short fast phase followed by a prolonged slow phase that eventually reached a plateau. The apparent EC_{50} value for 2-hydroxycarbazole induced Ca^{2+} release was found to be very similar for both skeletal muscle sarcoplasmic reticulum (200 ± 9 μM) and cardiac muscle sarcoplasmic reticulum (214 ± 24 μM), suggesting that 2-hydroxycarbazole mobilizes Ca^{2+} with similar affinities and by a similar mechanism in both muscle types. Maximal concentrations of 2-hydroxycarbazole (400–500 μM) were found to mobilize 10.2 ± 0.7% of the Ca^{2+} released by the ionophore A23187 in skeletal muscle sarco-plasmic reticulum and 6.9 ± 0.2% in cardiac muscle sarcoplasmic reticulum. The difference in the maximum amount of Ca^{2+} mobilized reflects the relative abundance of ryanodine receptors in the different muscle types, suggesting that the ryanodine receptor density per Ca^{2+} store is higher in skeletal muscle sarcoplasmic reticulum than in cardiac muscle sarcoplasmic reticulum. The release of Ca^{2+} induced by 2-hydroxycarbazole was highly cooperative in nature for both cardiac sarcoplasmic reticulum and skeletal muscle sarcoplasmic reticulum. Ca^{2+} release was also inhibited by either pre-treatment with caffeine or by both ruthenium red (30 μM) and tetracaine (1 mM). It has been demonstrated that both ruthenium red (Meissner et al., 1986) and tetracaine (Palade et al., 1989) are non-competitive inhibitors of Ca^{2+} release from the ryanodine receptor, suggesting that the mobilization of Ca^{2+} induced by 2-hydroxycarbazole was a direct result of ryanodine receptor activation. Ryanodine receptor type I is the most abundant isoform in skeletal muscle sarcoplasmic reticulum, while ryanodine receptor type II is the most abundant in cardiac muscle sarcoplasmic reticulum (McPherson and Campbell, 1993), suggesting that 2-hydroxycarbazole mobilized Ca^{2+} in a ryanodine receptor isoform independent fashion. The illustration that 2-hydroxycarbazole can mobilize Ca^{2+} from different isoforms of the ryanodine receptor is consistent with the mobilization of Ca^{2+} by caffeine. This would appear to suggest that 2-hydroxycarbazole activates the ryanodine receptor at the same site as caffeine and bromoeudistomin D. A similar site of action for ryanodine receptor activation by these pharmaco-logical agents is not surprising as they share a high degree of structural homology. Increasing concentrations of 2-hydroxycarbazole did not significantly affect [^{3}H]ryanodine binding to its receptor over the effective concentration range (0–500 μM 2-hydroxycarbazole). This contrasts with other activators such as caffeine and bromoeudistomin that increase specific [^{3}H]ryanodine binding (Takahashi et al., 1995a) and inhibitors such as ruthenium red and procaine that decrease specific [^{3}H]ryanodine binding (Imagawa et al., 1987). The activity of the skeletal muscle sarcoplasmic reticulum Ca^{2+} ATPase was slightly inhibited only by high concentrations of 2-hydroxycarbazole (400 μM and above). This study also showed that 2-hydroxycarbazole could mobilize Ca^{2+} from cerebellar microsomal stores and from testicular TM4 cells and therefore suggests that 2-hydroxycarbazole is capable of mobilizing Ca^{2+} from ryanodine receptors in a wide variety of tissues which contain these Ca^{2+} channels.

In summary, this work illustrates that 2-hydroxycarbazole is a novel pharmacological agent capable of Ca^{2+} mobilization via activation of the ryanodine receptor. The existence of an additional pharmacological agent capable of mobilizing Ca^{2+} through the ryanodine receptor provides an alternative to other Ca^{2+} mobilizing agents, and as such is useful for the further characterization of Ca^{2+} release from the ryanodine receptor. This study has shown that 2-hydroxycarbazole has the advantage of being ≈ 20 times more potent at mobilizing Ca^{2+} from the ryanodine receptor than caffeine. Although it is less potent than bromoeudistomin D, it has the advantage of being commercially available and cheap.

Acknowledgements

Both S.C.T. and C.L.L. contributed equally to this study. We would like to thank the British Heart Foundation for a PhD studentship to S.C.T and the BBSRC for a PhD studentship to C.L.L. In addition, we also thank The Wellcome Trust for financial support. Dr. S.C. Tovey is presently at the Laboratory of Molecular Signalling, The Babraham Institute, Babraham Hall, Babraham, Cambridge, CB2 4AT.

References

Brown, G.R., Sayers, L.G., Kirk, C.J., Michell, R.H., Michelangeli, F., 1992. The opening of the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} channel is inhibited by caffeine. Biochem. J. 282, 309–312.

Cheek, T.R., O'Sullivan, A.J., Moreton, R.B., Berridge, M.J., Burgoyne, R.D., 1990. The caffeine sensitive Ca^{2+} store in bovine adrenal chromaffin cells—an examination of its role in triggering secretion and Ca^{2+} homeostasis. FEBS Lett. 266, 91–95.

Cheek, T.R., Barry, V.A., Berridge, M.J., Missiaen, L., 1991. Bovine adrenal chromaffin cells contain an inositol 1,4,5-trisphosphate insensitive but a caffeine-sensitive Ca^{2+} store that can be regulated by intraluminal free Ca^{2+}. Biochem. J. 275, 697–701.

Endo, M., 1977. Calcium release from sarcoplasmic reticulum. Physiol. Rev. 57, 71–108.

Fleischer, S., Inui, M., 1989. Biochemistry and biophysics of excitation–contraction coupling. Annu. Rev. Biophys. Biophys. Chem. 18, 333–364.

Kobiyashi, J., Ishibashi, M., Nagai, U., Ohizumi, Y., 1989. 9-methyl-7-bromoeudistomin D a potent inducer of Ca^{2+} release from the sarcoplasmic reticulum of skeletal muscle. Experientia 45, 82–783.

Imagawa, T., Smith, J.S., Coronado, R., 1987. Purified ryanodine receptor from skeletal-muscle sarcoplasmic-reticulum is the Ca^{2+}-permeable pore of the calcium release channel. J. Biol. Chem. 262, 16636–16643.

Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q.-Y., Meissner, G., 1988. Purification and reconstitution of the calcium release channel from skeletal-muscle. Nature 331, 315–319.

McPherson, P.S., Campbell, K.P., 1993. Characterization of the major brain form of the ryanodine receptor Ca^{2+} release channel. J. Biol. Chem. 268, 13765–13768.

Meissner, G., Darling, E., Eveleth, J., 1986. Kinetics of rapid Ca^{2+} release by sarcoplasmic-reticulum—effects of Ca^{2+}, Mg^{2+} and adenine-nucleotides. Biochemistry 25, 236–244.

Meszaros, L.G., Bak, J., Chu, A., 1993. Cyclic ADP-ribose as an endogenous regulator of the non-skeletal type ryanodine receptor Ca^{2+} channel. Nature 364, 76–79.

Mezna, M., Michelangeli, F., 1997. Effects of thimerosal on the transient kinetics of inositol 1,4,5-trisphosphate-induced Ca^{2+} release from cerebellar microsomes. Biochem. J. 325, 177–182.

Michelangeli, F., 1990. Measuring Ca^{2+} uptake and inositol 1,4,5-trisphosphate-induced Ca^{2+} release in cerebellum microsomes using fluo-3. J. Fluoresc. 1, 203–206.

Michelangeli, F., Munkonge, F.M., 1990. Methods of reconstitution of the purified sarcoplasmic reticulum Ca^{2+}–Mg^{2+} ATPase using bile salt detergents to form membranes of defined lipid to protein ratios or sealed vesicles. Anal. Biochem. 194, 231–236.

Minta, A., Kao, J.P.Y., Tsien, R.Y., 1989. Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. J. Biol. Chem. 264, 8171–8178.

Nakamura, Y., Kobayashi, J., Gilmore, J., Mascal, M., Rinehart Jr., K.L., Nakamura, H., Ohizumi, Y., 1986. Bromo-eudistomin-D, a novel inducer of calcium release from fragmented sarcoplasmic-reticulum that causes contractions of skinned muscle-fibers. J. Biol. Chem. 261, 4139–4142.

Palade, P., Dettbarn, C., Brunder, D., Stein, P., Hals, G., 1989. Pharmacology of calcium release from sarcoplasmic reticulum. J. Bioenerg. Biomembr. 21, 295–320.

Parker, I., Ivorra, I., 1991. Caffeine inhibits inositol trisphosphate-mediated liberation of intracellular calcium in *Xenopus* oocytes. J. Physiol. (London) 433, 229–240.

Rousseau, E., Liu, Q.-Y., Meissner, G., 1988. Activation of the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum by caffeine and related compounds. Arch. Biochem. Biophys. 267, 75–86.

Siato, A., Seiler, S., Chu, A., Fleischer, S., 1984. Sarcoplasmic-reticulum terminal cisternae preparation and morphology of from rabbit skeletal-muscle. J. Cell Biol. 99, 875–885.

Seino, A., Kobayashi, M., Kobayashi, J., Fang, Y.-I., Ishibashi, M., Nakamura, H., Momose, K., Ohizumi, Y., 1991. 9-methyl-7-bromoeudistomin D: a powerful radiolabable Ca^{2+} releaser having caffeine-like properties acts on Ca^{2+}-induce Ca^{2+} release channels in sarcoplasmic reticulum. J. Pharmacol. Exp. Ther. 256, 861–867.

Takahashi, Y., Furukawa, K.-I., Kozutsumi, D., Ishibashi, M., Kobayashi, J., Ohizumi, Y., 1995a. Structure-activity relationship of bromoeudistomin-D, a powerful Ca^{2+} releaser in skeletal-muscle sarcoplasmic-reticulum. Eur. J. Pharmacol. 288, 285–293.

Takahashi, Y., Furukawa, K.-I., Kozutsumi, D., Ishibashi, M., Kobayashi, J., Ohizumi, Y., 1995b. 4,6-Dibromo-3-hydroxycarbazole (an analog of caffeine-like Ca^{2+} releaser), a novel type of inhibitor of Ca^{2+}-induced Ca^{2+} release in skeletal-muscle sarcoplasmic-reticulum. Br. J. Pharmacol. 114, 941–948.

Tovey, S.C., Godfrey, R.E., Hughes, P.J., Mezna, M., Minchin, S.D., Mikoshiba, K., Michelangeli, F., 1997. Identification and characterization of inositol 1,4,5-trisphosphate receptors in rat testis. Cell Calcium 21, 311–319.

Walton, P.D., Airey, J.A., Sutko, J.L., Beck, C.F., Mignery, G.A., Sudhof, T.C., Deerinck, T.J., Ellisman, M.H., 1991. Ryanodine and inositol trisphosphate receptors coexist in avian cerebellar Purkinje neurons. J. Cell. Biol. 113, 1145–1157.