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Bis(2-hydroxy-3-tert-butyl-5-methyl-phenyl)-methane (bis-phenol) is a potent and selective inhibitor of the secretory pathway Ca^{2+} ATPase (SPCA1)

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ABSTRACT

The secretory pathway Ca^{2+} ATPase (SPCA) provides the Golgi apparatus with a Ca^{2+} supply essential for Ca^{2+}-dependent enzymes involved in the post-translational modification of proteins in transit through the secretory pathway. Ca^{2+} in the Golgi apparatus is also agonist-releasable and plays a role in hormone-induced Ca^{2+} transients. Although the Ca^{2+} ATPase inhibitors thapsigargin is more selective for the sarcoplasmic–endoplasmic reticulum Ca^{2+} ATPase (SERCA) than for SPCA, no inhibitor has been characterised that selectively inhibits SPCA.

A number of inhibitors were assessed for their selectivity to the human SPCA1d compared to the more ubiquitous human SERCA2b. Each isoform was over-expressed in COS-7 cells and the Ca^{2+}-dependent ATPase activity measured in their microsomal membranes. Both bis(2-hydroxy-3-tert-butyl-5-methyl-phenyl)methane(bis-phenol) and 2-aminoethoxydiphenylborate (2-APB) selectively inhibited SPCA1d (with IC_{50} values of 0.13 μM and 0.18 mM, respectively), which were of 62- and 8.3-fold greater potency than the values for hSERCA2b (IC_{50} values; 8.1 μM and 1.5 mM, respectively). Other inhibitors tested such as bis-phenol-A, tetrabromobisphenol-A and trifluoperazine inhibited both Ca^{2+} ATPases similarly. Furthermore, bis-phenol was able to mobilize Ca^{2+} in cells that had been pre-treated with thapsigargin. Therefore we conclude that given the potency and selectivity of bis-phenol it may prove a valuable tool in further understanding the role of SPCA in cellular processes.

1. Introduction

The secretory pathway Ca^{2+} ATPase (SPCA) is a primary active Ca^{2+} transporter that is located on the Golgi apparatus and loads this organelle with Ca^{2+} [1]. Ca^{2+} is required to modulate the activities of enzymes located in this organelle that are required to post-translationally modify and control the folding of newly synthesised proteins, such as rat thyroglobulin [2] and human matrix γ-carboxyglutamic acid protein [3]. In rat mammary glands, Ca^{2+} in the Golgi apparatus is also secreted with milk during lactation [4]. Previous studies in our laboratory has shown that significant levels of Ca^{2+} uptake in a variety of tissues such as brain, vascular smooth muscle and testes is driven through SPCA [5]. In fact SPCA is also able to influence Ca^{2+}-mediated hormone responses because the Golgi Ca^{2+} store is releasable by stimulation with agonists, such as arginine vasopressin and interleukin-8 via IP_3-sensitive Ca^{2+} channels [6,7]. The contribution that SPCA makes to shaping the dynamics of Ca^{2+} transients in signalling cascades has also been noted in the process of glucose-stimulated insulin secretion in rats [8]. SPCA is mainly located on the trans-Golgi compartment of mammalian cells [1] and recently, it has been shown that Ca^{2+} release from this store in rat neonatal cardiac myocytes can also be induced through the activation of ryanodine receptors (RyRs) [9].

Unlike its closely related intracellular localized ER Ca^{2+} ATPase, the sarcoplasmic–endoplasmic reticulum Ca^{2+} ATPase (SERCA), there has yet to be an inhibitor that has been fully characterised and shown to be selective for SPCA over that for SERCA. Thapsigargin (a highly selective SERCA inhibitor), has been widely used to elucidate the role of both SERCA and ER Ca^{2+} mobilization in cellular processes [10]. Thapsigargin has proved to be considerably less potent towards other Ca^{2+} ATPase such as SPCA [1,11]. Therefore it would be of considerable benefit to identify an inhibitor that is selective for SPCA in order to aid the further understanding of the role SPCA and the Golgi Ca^{2+} store has in regulating cellular processes.

The aim of this study is to identify SPCA inhibitors that are able to selectively distinguish between the two types of intracellular Ca^{2+} ATPases, SPCA and SERCA, and additionally be selective towards SPCA. In this study the human forms of these two Ca^{2+} ATPases (hSPCA1d and hSERCA2b) were used because they represent the most abundant isoforms and splice variant of both, as well as containing all possible structural features as they are the largest spliced variants forms of each Ca^{2+} ATPase.

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To date, many tens (if not hundreds) of SERCA inhibitors have been identified [10] and a selection of these were tested on both hSPCA1d and SERCA2b Ca^{2+} ATPase in order to identify any that were potentially selective towards SPCA.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) (supplemented with 5.5 mM glucose) media, Dulbecco's phosphate buffered saline (DPBS) and trypsin–Versene solution were purchased from Lonza. Foetal bovine serum (FBS), L-glutamine and penicillin-streptomycin (for media supplementation) were purchased from PAA. Genejuice transfection reagent was purchased from Novagen. Thapsigargin, 2-aminophenylborate (2-APB), bis-phenol-A (BPA), tetrabromobisphenol-A (TBBPA) and trifluoperazine (TFP) were purchased from Sigma. bis(2-hydroxy-3-tert-butyl-5-methyl-phenyl)-methane (CAS number 119-47-1; also known as 2,2′-methylenebis(6-tert-butyl-4-methylphenol) and commonly referred to as Ralox 46 or Bis-phenol not bis-phenol-A) was purchased from Pfaltz & Bauer. Ionophore A23187 was purchased from Calbiochem. Anti-SPCA1 was a custom-made antibody from BioCarta GmbH [5,7] and anti-SERCA2 (clone ILD8) was purchased from Abcam. Immobilon Western HRP substrate was purchased from Millipore. All other chemicals were of analytical grade. Expression plasmids containing the coding sequence for hSPCA1d (hSPCA1d-pMT2) and hSERCA2b (hSERCA2b-pMT2) were both gifts from Dr. J. Vanoevelen (Katholieke Universiteit Leuven, Belgium).

2.2. Cell culture & transfection

COS-7 cells (a gift from Dr. N. Hotchin (University of Birmingham, UK) were cultured in DMEM media, supplemented with 10% v/v FBS, 2 mM L-glutamine and 1% v/v penicillin-streptomycin. A7r5 vascular smooth muscle cells (VSMs) cells were cultured in DMEM media with 10% FBS, 4 mM L-glutamine, 1% non-essential amino acids and 1% v/v penicillin–streptomycin as described in [7].

For transfection, the COS-7 cells were seeded at a density of 1.2×10^6 cells per 10 cm diameter Petri dish and Genejuice transfection reagent with plasmid DNA was used following manufacturer's instructions. Transfection was done 24 h after seeding cells onto Petri dishes. Both transfected cells and control non-transfected cells were used 72 h after seeding onto Petri dishes for microsomal membrane preparation.

2.3. Microsomal membrane preparation

The method used was as described in a previous publication [7]. Essentially the cells were homogenized using a Polytron homogenizer followed by centrifugation at 20,000g (for 15 min.) and the supernatant obtained from this spin was further centrifuged at 100,000 g for 50 min. The pellet was then re-suspended in fresh buffer and stored at −80 °C.

2.4. Western blotting

The method used was as described in a previous publication [5,7]. Anti-SPCA1 (previously raised and used as described in [5,7]) and anti-SERCA2 antibodies were used at dilution ratios of 1:80 and 1:2500, respectively.

2.5. Ca^{2+}-dependent ATPase activity measurements

The method was as described in a previous publication (and which included both 2 μM vanadate and 2 mM azide to inhibit any contribution from the plasma membrane Ca^{2+} ATPase (PMCA) and mitochondrial Ca^{2+} uptake, respectively) [7,12], but with the following modifications. The reaction was initiated by the addition of 6 mM ATP and the rate was measured over 45 min (with the activity being linear for at least 75 min). Each assay also contained 10 μg of microsomal membrane protein. Thapsigargin, BPA, bisphenol, 2-APB, TBBPA and TFP were all prepared in DMSO, which was used as a vehicle and that the concentration of DMSO used was <1% v/v, which caused little or no effect on activity.

2.6. Intracellular [Ca^{2+}] measurements

It has previously been shown that aorta and vascular smooth muscle cells, A7r5 VSMCs express relatively high levels of SPCA [5,7], therefore intracellular Ca^{2+} measurements where undertaken on these cells using Ca^{2+} imaging fluorescence microscopy as described previously [7] but with the following modifications: Fluo-3 AM (Sigma) was used at a final concentration of 7.5 μM. Bis-phenol (dissolved in DMSO) or thapsigargin (1 μM) were added to cells 1 min after initiating recording. In order to focus on changes in cytosolic Ca^{2+} concentrations occurring from internal Ca^{2+} stores, prior to initiating the recordings, the Hank's buffered saline solution was supplemented with 1.26 mM EGTA such that the cells were in the absence of extracellular Ca^{2+}.

3. Results

3.1. SPCA1 & SERCA2 expression levels and activity in hSPCA1d/hSERCA2b-pMT2-transfected & non-transfected COS-7 cells

Western blotting was used to monitor transfection efficiency of COS-7 cells (Fig. 1). Microsomal membranes from COS-7 cells transfected to over-express hSPCA1d and hSERCA2b produced highly detectable levels of SPCA1 and SERCA2 protein, respectively. Under the same experimental conditions, by comparison, SPCA1 and SERCA2 protein expression were both only detected at very low amounts in non-transfected cell membranes.

The phosphate liberation assay method was used to measure Ca^{2+}-dependent ATPase activities in microsomal membranes isolated from hSPCA1d and hSERCA2b over-expressing COS-7 cells.

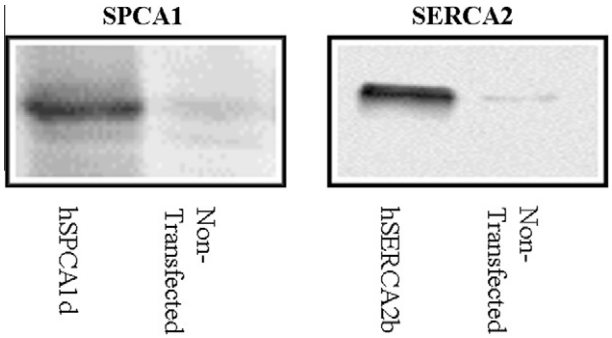

Fig. 1. Immuno-detection of SPCA1 & SERCA2 expression in hSPCA1d/hSERCA2b-pMT2-transfected & non-transfected COS-7 cells. Twenty μg of total protein in each sample of microsomal membranes was separated by electrophoresis for Western blotting. These samples were probed with either anti-SPCA1 or anti-SERCA2 to visualise the amount of Ca^{2+}-ATPase protein present in microsomal membranes from cells that had been transfected to over-express hSPCA1d and hSERCA2b, respectively, and compared to control membranes from non-transfected cells. The blots are representative of three replicate experiments.

Activities were also measured from microsomes isolated from non-transfected COS-7 cells as a control and these values (which constituted less than 15% of the overall activity) were subtracted from the activities determined for the transfected cell microsomal membranes. The Ca^{2+} ATPase activity values from three separate membrane preparations for both hSPCA1d and hSERCA2b, measured in the absence and presence of 1 μM thapsigargin. Ca^{2+} ATPase activity from hSPCA1d cells measured in the absence and presence of 1 μM thapsigargin did not differ significantly (24.7 ± 1.9 and 21.2 ± 4.3 nmol/min/mg respectively). Whereas the activity values from hSERCA2b expressing cells, in the presence of 1 μM thapsigargin were inhibited by >95% compared to its absence (i.e. in the absence of thapsigargin the activity was 14.7 ± 1.7 nmol/min/mg and in the presence of thapsigargin the activity was 0.7 ± 0.5 nmol/min/mg) and this clearly demonstrates that hSPCA1d is insensitive to inhibition by thapsigargin at this concentration [11]. All activity measurements with hSPCA1d were therefore undertaken in the presence of 1 μM thapsigargin.

3.2. Effects of a number of inhibitors on hSPCA1d & hSERCA2b Ca^{2+} ATPase activity

Bis-phenol, BPA, 2-APB, TBBPA and TFP were added at varying concentrations to hSPCA1d and hSERCA2b microsomal membranes and their activities measured. The results showed that all five compounds inhibited both hSPCA1d and hSERCA2b activity. Table 1 shows the IC_{50} values that were calculated for each compound and for both Ca^{2+} ATPases. From this, it was noted that bis-phenol (Fig. 2) and TBBPA were the most potent at inhibiting both hSPCA1d and hSERCA2b activity with IC_{50} values in the low to sub-μM concentration range.

When comparing the IC_{50} values obtained for both hSPCA1d and hSERCA2b for the inhibitors bis-phenol and 2-APB it was clear that they were both more selective for hSPCA1d than for hSERCA2b, respectively (see Fig. 3). In contrast, the difference in inhibition potency between the two Ca^{2+} ATPases for TBBPA (1.3-fold) and TFP (0.6-fold) and BPA (1.8-fold) were not as significant (Table 1).

3.3. Effects of bis-phenol on intracellular [Ca^{2+}] levels

In order to assess the effects of bis-phenol on intracellular [Ca^{2+}] levels, A7r5 cells were used as they expressed naturally abundant levels of SPCA [7]. It was felt that artifactual results could potentially be generated in cells that were transfected to over-express SPCA both to unnatural levels and unnatural locations within the cell. Due to differences in the potency of bis-phenol for SPCA and SERCA, the action of both 2 μM and 20 μM caused increases in intracellular [Ca^{2+}] levels. Fig. 3 shows the effects of bis-phenol 20 μM on intracellular [Ca^{2+}] levels in A7r5 VSM cells after treatment with 1 μM thapsigargin to remove any contribution from SERCA-loaded Ca^{2+} stores. As can be seen even once SERCA-loaded Ca^{2+} stores were depleted, bis-phenol was still able to release additional Ca^{2+} from these cells, indicating that bis-phenol mobilizes Ca^{2+} from a thapsigargin-insensitive Ca^{2+} pool, presumably loaded by SPCA.

Table 1
IC_{50} values of hSPCA1d & hSERCA2b inhibitors. IC_{50} values ± S.E.M (n = 3) for each of the inhibitors tested. Values for bis-phenol and 2-APB were calculated from the data presented in Fig. 3.

Inhibitor	IC_{50} values for hSPCA1d (μM)	IC_{50} values for hSERCA2b (μM)	Fold difference (IC_{50} SERCA/IC_{50} SPCA)
Bis-	phenol	0.13 ± 0.01	8.10 ± 0.41
62			
2-APB	180 ± 5	1500 ± 200	8.3
TBBPA	0.54 ± 0.08	0.71 ± 0.07	1.3
TFP	20.0 ± 0.9	11.0 ± 3.1	0.6
BPA	130 ± 6	230 ± 30[a]	1.8

[a] Data for BPA was determined for SERCA1a and previously published in [16].

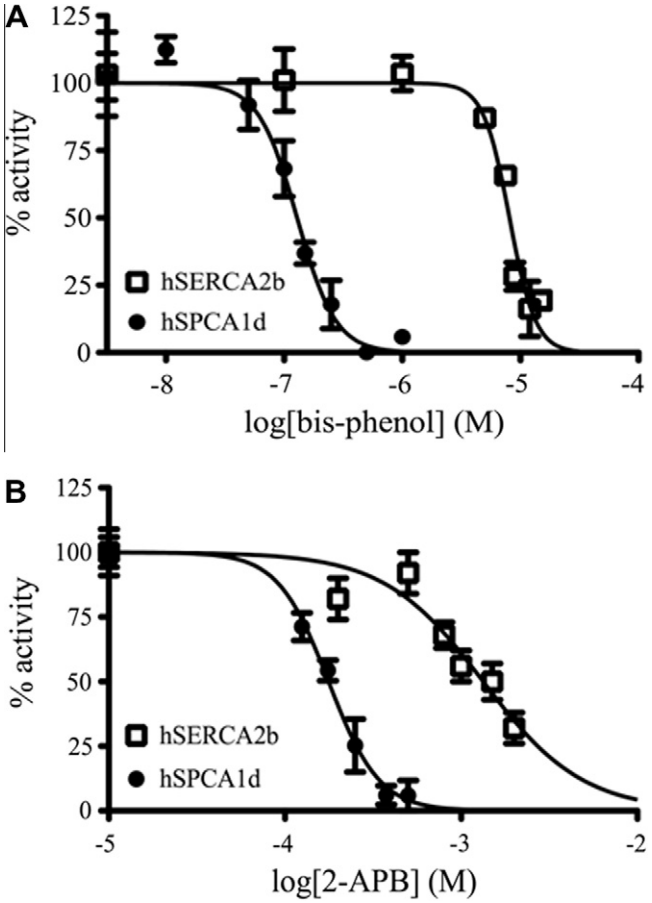

Fig. 2. Activity curves for inhibition of hSPCA1d & hSERCA2b by bis-phenol & 2-APB. Percentage activity was calculated for (A) bis-phenol and (B) 2-APB. Mean activities were measured from three replicate assays for each concentration of compound tested. Error bars indicate S.E.M values. Activities for hSPCA1d (●) membranes were undertaken in the presence of 1 μM thapsigargin and those for hSERCA2b (□) membranes where not. The data for 2-APB inhibition of hSERCA2b was reproduced from a previous publication [12]. The R^2 values for the goodness-of-fit for the inhibition plots were ⩾0.9.

4. Discussion

Our study has shown that bis-phenol can potently inhibit hSPCA1d (IC_{50} 0.13 μM) with 62-fold greater potency than hSERCA2b (IC_{50} 8.1 μM). Earlier studies have shown that the IC_{50} for bis-phenol on the purified rabbit SERCA1a was 2–3 μM [13], which may indicated a slight variability in SERCA-isoform sensitivity for this inhibitor. In experiments undertaken with SERCA it was determined that bis-phenol caused inhibition by slowing down the E2 to E1 transition step in the mechanism of the ATPase [13]. Given the likely similarity in both the structures of SPCA and SERCA, in addition to their kinetic/mechanistic similarities [11], it would also be likely that bis-phenol inhibits SPCA in a similar manner to SERCA, however, detailed kinetic analysis would need to be undertaken to confirm this.

Our studies therefore show bis-phenol to be suitable in differentiating the effects of SPCA from that of SERCA, as well as from

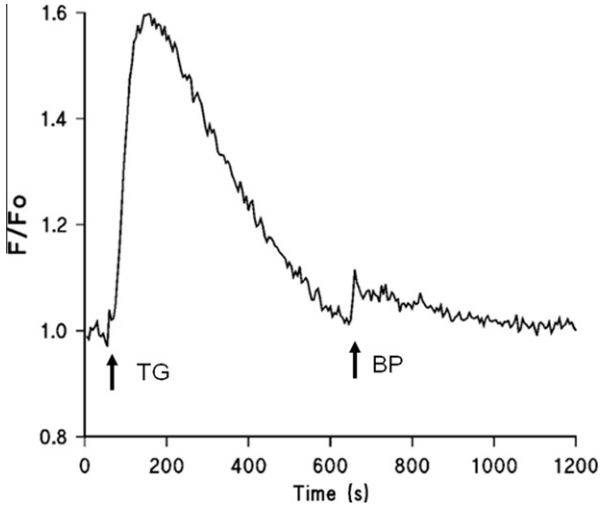

Fig. 3. Effects of thapsigargin and bis-phenol on intracellular [Ca^{2+}] levels in A7r5 cells. Ca^{2+} imaging was undertaken by monitoring changes in fluorescence of fluo-3-loaded A7r5 cells using a fluorescence microscope. The fluorescence intensity for 10 cells within the FOV was monitored over a period of 20 min and averaged to produce the trace shown. The addition of 1 μM thapsigargin (TG) followed by 20 μM bis-phenol (BP) are indicated by the arrows.

a previous study PMCA (for which bis-phenol has an IC_{50} value of 9 μM) [13].

In order to determine its suitability for use in intact cells, we have previously shown that it can induce Ca^{2+} mobilization from internal stores of intact HL-60 cells at low μM concentrations [13]. In addition, in this current study we have now demonstrated that low concentrations of bis-phenol can cause Ca^{2+} mobilization in A7r5 cells even after SERCA-loaded Ca^{2+} stores have been depleted by thapsigargin treatment. The reason that only a relatively small change in Ca^{2+} was observed upon bis-phenol treatment could be due to the fact that in some cells SPCA-located Ca^{2+} stores also shows some co-localisation with Ryanodine receptors (RyRs) which are themselves Ca^{2+} sensitive [9]. Therefore upon a substantial rise in intracellular [Ca^{2+}] due to thapsigargin treatment this will likely also trigger Ca^{2+}-induced Ca^{2+} release from those store that contain both SPCA and RyRs, thus reducing the amount of Ca^{2+} mobilisation seen when bis-phenol is then added. Additionally, the Golgi membrane could also be less leaky to Ca^{2+} compared to the ER and therefore only a small amount of Ca^{2+} is released. In order to confirm these possibilities intra-luminal Golgi [Ca^{2+}] levels would need to be monitored using Golgi targeted Ca^{2+} indicating proteins such as those used in [9].

2-APB can also selectively inhibit hSPCA1d over hSERCA2b, with 8.3-fold greater potency, but due to its low potency and its ability to affect other Ca^{2+} transporters such as IP_3-sensitive Ca^{2+} channels [14] and store-operated Ca^{2+} channels [15], its usefulness would be rather limited.

In summary, bis-phenol may likely prove useful as a pharmacological tool in studies of the role of SPCA in regulating both agonist-inducible Ca^{2+}release from the Golgi apparatus in addition to the role Ca^{2+} plays in regulating processes occurring in this organelle.

Acknowledgments

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