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Characterisation of a novel Ca^{2+} pump inhibitor (bis-phenol) and its effects on intracellular Ca^{2+} mobilization

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Abstract

Bis-phenol, a phenolic antioxidant, is an inhibitor of sarcoplasmic reticulum (SR), endoplasmic reticulum (ER) and plasma membrane Ca^{2+} ATPases. The concentration of bis-phenol giving half-maximal inhibition of the SR Ca^{2+}-ATPase is 2 μM. On binding to the SR Ca^{2+}-ATPase it shifts the E_2 to E_1 transition towards the E_2 state and slows the transition between E_2 to E_1. Bis-phenol completely inhibits Ca^{2+}-dependent ATP hydrolysis and Ca^{2+} uptake by rat cerebellar microsomes at a concentration of 30 μM. The plasma membrane Ca^{2+}-ATPase is also completely inhibited at similar concentrations, however, the Na^+/K^+-ATPase is only marginally affected. Other inhibitors of the ER Ca^{2+}-ATPases, thapsigargin and 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (BHQ), inhibit Ca^{2+} uptake by approximately 75%. Bis-phenol therefore inhibits all types of ER Ca^{2+}-ATPases present in cerebellum. This inhibitor is also able to mobilize Ca^{2+} from intracellular Ca^{2+} stores, including those sensitive to $InsP_3$, in intact HL-60 cells.

1. Introduction

Specific inhibitors of the Ca^{2+}-ATPase of endoplasmic reticulum (ER) have proved very useful tools in probing the molecular mechanisms of calcium homeostasis [1–6]. The inhibitors thapsigargin and 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (BHQ) are most commonly used as they are plasma membrane-permeable and therefore able to inhibit the Ca^{2+} pump in the ER of intact cells [1–3]. Studies with permeabilised cells and microsomes have suggested that thapsigargin and BHQ inhibit only a subpopulation of intracellular Ca^{2+} stores as approximately 20–30% of Ca^{2+} uptake remains after thapsigargin or BHQ treatment [4–6]. By contrast, thapsigargin completely inhibits the Ca^{2+}-ATPase of SR [7–10].

At least two isoforms of the Ca^{2+}-ATPase exist in non-muscle cells: the SERCA2b isoform (similar to the cardiac isoform except for an extra 49 or 50 amino acids at the carboxy terminus) and the SERCA3 isoform which has a 75% sequence homology to the SERCA1 (skeletal muscle isoform) and SERCA2 isoforms [11–13]. Recent studies on Ca^{2+}-dependent phosphorylation of the nonmuscle isoforms has suggested that of the two types present in platelets and other cell types, one is sensitive to thapsigargin as determined from a decrease in Ca^{2+}-dependent phosphorylation by ATP, whilst the other is insensitive to thapsigargin but sensitive to BHQ [14,15]. These findings indicate the presence of two distinct types of Ca^{2+}-ATPase in endoplasmic reticulum in a variety of cell types which differ in their sensitivities to these inhibitors. These observations may well explain the heterogeneity in calcium handling properties of Ca^{2+} stores with some stores being insensitive to thapsigargin and BHQ inhibition. When studying Ca^{2+} stores in permeabilised or intact cells, it would be beneficial to complement the use of thapsigargin and BHQ with other membrane permeable inhibitors of the Ca^{2+}-ATPase which are able to inhibit all Ca^{2+} pumps present in cells in order to differentiate between the different Ca^{2+} stores.

Thapsigargin and BHQ have been shown to affect a number of steps in the reaction sequence of the Ca^{2+}-

Abbreviations: bis-phenol, bis(2-hydroxy-3-*tert*-butyl-5-methylphenyl)methane; $InsP_3$, inositol 1,4,5-trisphosphate; FITC, fluorescein 5′-isothiocyanate; BHQ, 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone; BHT, 3,5-dibutyl-4-hydroxytoluene; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; IC_{50}, concentration which causes half-maximal response.

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ATPase, including decreases in the levels of both ATP- and P_i-dependent phosphorylation and stabilization of the E_2 conformational state of the ATPase [7–9]. Here we investigate the mechanism of action of bis-phenol, a novel inhibitor of the skeletal muscle Ca^{2+}-ATPase [27] and assess its usefulness as an inhibitor of non-muscle Ca^{2+}-ATPases in both isolated miscrosomes and intact cells.

2. Material and methods

Bis-phenol was purchased from Pfaltz and Bauer (Flushing, NY), fluorescein 5'-isothiocyanate (FITC) and fluo-3 from Molecular Probes, and all other reagents from Sigma. Skeletal muscle SR was prepared and rates of Ca^{2+}-dependent ATP hydrolysis were measured as described Michelangeli and Munkonge [16]. From Coomassie-stained polyacrylamide gel electrophoresis our SR preparations contain typically 70–80% Ca^{2+}-ATPase [16]. In some experiments the free Ca^{2+} concentration was varied from 50 nM to 3 mM by altering the EGTA and Ca^{2+} concentrations; free Ca^{2+} concentrations were calculated using the binding constants for Mg^{2+} and Ca^{2+} to EGTA and ATP given in Gould et al. [17]. Microsomes from rat cerebella and rat kidney medulla were prepared as described in Michelangeli et al. [18,20]. Human red blood cell membranes were prepared and measurements of Ca^{2+}-dependent ATP hydrolysis were performed as described in Thastrup et al. [1] and Brown et al. [19], respectively. Ca^{2+} uptake measurements were carried out using fluo-3 as described in [20,21]. Na^+/K^+-ATPase activities were measured using an enzyme coupled assay in a buffer containing 40 mM Hepes-Tris, 5 mM ATP, 10 mM $MgSO_4$, 100 mM NaCl, 10 mM KCl, 1 mM EGTA, 0.01% Triton X-100, 0.36 mM NADH, 2 mM PEP, 18 units lactate dehydrogenase, 7.5 units pyruvate kinase (pH 7.2), in the absence and presence of 2.0 mM ouabain.

Measurements of the E_1–E_2 equilibrium were determined by measuring changes in fluorescence of the Ca^{2+}-ATPase labelled with FITC as described by Michelangeli et al. [22] and Froud and Lee [23]. SR was labelled with FITC to give a molar ratio of FITC/ATPase of approximately 0.5:1, as follows. SR (1 mg) was incubated in 100 μl of 1 M KCl, 50 mM potassium phosphate, 0.25 M sucrose (pH 8) to which was added 2.5 nmol FITC from a stock solution of FITC in dry dimethylformamide (5 mM). The reaction was left to stand for 1 h at room temperature. 250 μl of 0.2 M sucrose, 50 mM Tris-HCl (pH 7.0) was added and the mixture left on ice until use. Labelled SR (10 μg) was added to 2.5 ml of 50 mM Tris, 50 mM maleate, 5 mM $MgSO_4$, 100 mM KCl, 0.1 mM EGTA (pH 6 or pH 7), 25°C and the fluorescence intensity monitored with a Perkin-Elmer LS 50 spectrofluorimeter (exciting at 495 nm and detecting the emission at 525 nm). The change in FITC fluorescence was measured upon addition of 0.4 mM $CaCl_2$.

Measurements of ATP-dependent phosphorylation were as described in [22]. The EP_{max} level of the SR used was found to be 1.6 nmol/mg. We have previously shown that EP_{max} levels in SR and purified Ca^{2+}-ATPase vary between preparations and relate to the proportion of ATPase damaged during isolation [35]. HL-60 cells were grown in RPMI 1640 culture medium supplemented with 5% foetal calf serum (Gibco). The cells were loaded with fura-2 by incubation in culture medium containing 5 μM fura-2AM (Sigma), at 37°C for 30 min. The cells were then washed and resuspended at a concentration of 0.5 million cells/ml in Hanks balanced salt medium (pH 7.4). 2 ml of cells were added to a stirred cuvette at 37°C and fura-2 fluorescence was measured using a Perkin-Elmer LS-50 spectrofluorimeter (excitation 340 nm, emission 510 nm) and fluorescence changes were related to intracellular Ca^{2+} concentrations as described by Grynkiewicz et al. [24]. Some intracellular calcium measurements were carried out in the presence of 5 mM EGTA to remove external free Ca^{2+}, so that the changes observed were due only to mobilization from internal Ca^{2+} stores.

3. Results

Fig. 1 shows the inhibition of the Ca^{2+}-ATPase activity of the skeletal muscle sarcoplasmic reticulum caused by bis-phenol at 37°C (pH 7.2); half-maximal inhibition was observed at 2 μM bis-phenol.

Fig. 2A shows the typical bell-shaped dependence of ATPase activity on free Ca^{2+} concentration. Maximum ATPase activity was observed at 1–6 μM free Ca^{2+}, which was not substantially altered in the presence of 2

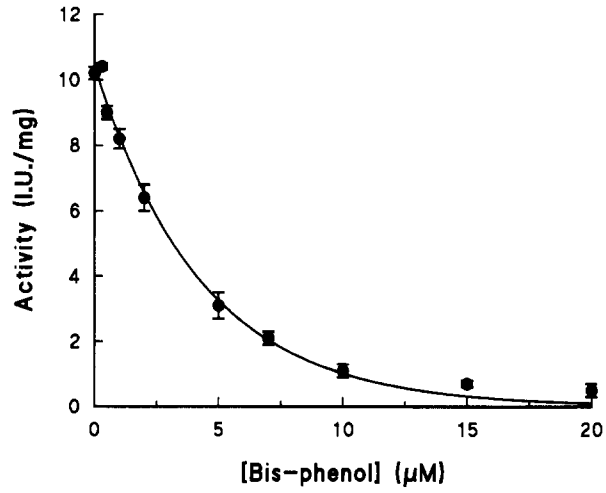

Fig. 1. The effects of bis-phenol on SR Ca^{2+}-ATPase activity. Bis-phenol was preincubated for 10 min with SR (0.025 mg) in assay buffer (40 mM Hepes-KOH, 2.1 mM ATP, 0.53 mM PEP, 0.15 mM NADH, 1.01 mM EGTA, 7.5 I.U. pyruvate kinase, 18 I.U. lactate dehydrogenase (pH 7.2) and 37°C). ATPase activity was measured in the presence of A23187 (10 μg/ml) by the addition of 1 μM free Ca^{2+}. The data are presented as the means ± S.E. of three determinations.

μM bis-phenol, although the V_{max} was reduced by approx. 50%. Neither the K_m values for the high-affinity (activatory) Ca^{2+} sites (0.5 μM) nor for the low-affinity (inhibitory) sites (0.16 mM) were affected by bis-phenol (Fig. 2A). Binding of Ca^{2+} to the Ca^{2+}-ATPase is known to be highly co-operative [23]; Hill plots of the data for the activation phase gave a Hill coefficient of 1.6 which again was unaffected by 2 μM bis-phenol.

Fig. 2B shows the dependence of ATPase activity on the concentration of ATP in the presence and absence of bis-phenol. The dependence of ATPase activity on ATP concentration is complex and can be fitted to a modified form of the Michaelis-Menten equation (see Ref. [25]) assuming that ATP interacts at two sites: a high-affinity (catalytic) site and a low-affinity (regulatory) site [17,18,25]. The activity profile in the absence of bis-phenol was fitted assuming K_m and V_{max} values for the high-affinity site of 4.3 μM and 4.7 I.U./mg, respectively and K_m and V_{max} values for the low-affinity site of 1.5 mM and 6.3 I.U./mg, respectively. As shown in Fig. 2B addition of 2 μM bis-phenol had little effect on ATPase activity at low ATP concentrations but substantially inhibited at higher ATP concentrations. In the presence of 2 μM bis-phenol, the data could be fitted to a normal Michaelis-Menten equation with K_m and V_{max} values of 4.8 μM and 4.6 I.U./mg, respectively.

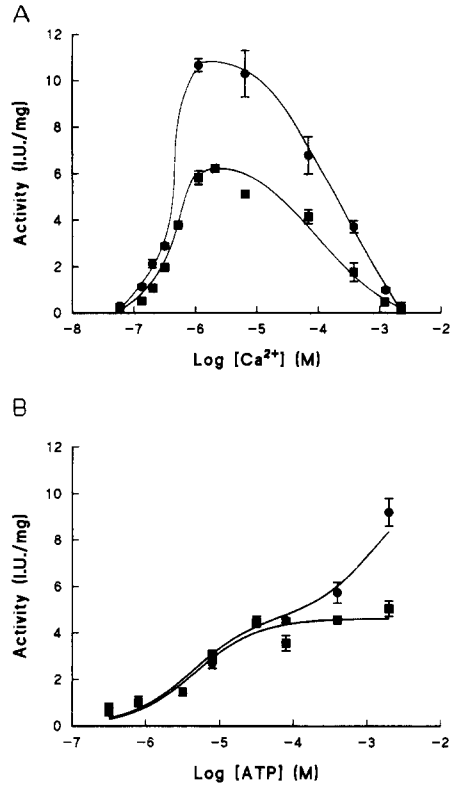

Fig. 2. The effects of bis-phenol on SR Ca^{2+}-ATPase activity as a function of the concentrations of free Ca^{2+} and ATP. ATPase activity was measured at pH 7.2, 37°C as described in the legend of Fig. 1. The SR was preincubated in the absence (●) and presence (■) of 2 μM bis-phenol, and the activities measured as a function of (A) free [Ca^{2+}] and (B) [ATP]. The K_m and V_{max} for the high- and low-affinity Ca^{2+} sites were estimated graphically from plot 2A. The K_m and V_{max} for the catalytic and regulatory ATP sites were determined by fitting the data in plot 2B to a modified version of the Michaelis-Menten equation given in Ref. [25]. The data are presented as the means ± S.E. of 3–5 determinations.

Table 1
The effects of bis-phenol on phosphoenzyme formation by [γ-^{32}P]ATP

[ATP] (μM)	EP/EP_{max}	
	0 μM bis-phenol	5 μM bis-phenol
1.0	0.42 ± 0.04	0.38 ± 0.05
3.0	0.61 ± 0.11	0.72 ± 0.06
10.0	1.06 ± 0.06	0.92 ± 0.09
30.0	1.00 ± 0.10	1.05 ± 0.09

Phosphorylations were carried out with SR (0.1 mg/ml) in buffer containing 40 mM Hepes-Tris, 100 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.2, 25°C. Maximum levels of phosphorylated protein (EP_{max}) was determined to be 1.6 nmol/mg. The data present the means ± S.E. of three determinations.

Table 1 shows the effect of bis-phenol on the maximal level of phosphorylation of the ATPase by [γ-^{32}P]ATP under conditions (1 mM Ca^{2+}) where the rate of dephosphorylation would be expected to be low. As shown, 5 μM bis-phenol has little effect on maximal levels of phosphorylation.

It has been shown recently that inhibitors such as nonylphenol, thapsigargin and BHQ affect the E_1–E_2 equilibrium of the ATPase [9,22]. This equilibrium can be conveniently monitored from changes in the fluorescence of FITC-labelled ATPase [22,23]. It has been shown that addition of Ca^{2+} results in a decrease in fluorescence intensity that can be attributed to the E_2 to E_1 conformational change; the fluorescence change observed at pH 6.0 is greater than that at pH 7.0 (see Fig. 3), attributable to a pH-dependence of the E_2–E_1 equilibrium, E_2 being favoured at low pH [23]. The fluorescence response to Ca^{2+} is larger in the presence of bis-phenol than in its absence, particularly at pH 7.0 (Fig. 3) consistent with a shift in the E_2–E_1 equilibrium towards E_2. The decrease in fluorescence intensity observed on addition of Ca^{2+} in the absence of bis-phenol is relatively rapid and cannot be conveniently followed in a conventional fluorimeter. However, in the presence of bis-phenol the fluorescence change becomes significantly slower with a half-life of 11 s in the presence of 3.5 μM bis-phenol at pH 6 (Fig. 3).

The microsomal fraction of cerebellum has been shown to be relatively abundant in ER Ca^{2+}-ATPases [18,20]. Fig. 4 shows the effects of bis-phenol on the rat cerebellar ER Ca^{2+}-ATPase activity. Activity is completely inhibited at 30 μM bis-phenol, with half maximal inhibition (IC_{50}) occurring at 12 μM. Fig. 4 also shows that the plasma membrane Ca^{2+}-ATPase from red blood cell membranes and to a much lesser degree the Na^+/K^+-ATPase from kidney medulla are also inhibited. Fig. 5 shows that Ca^{2+} uptake into cerebellar microsomes is also inhibited by

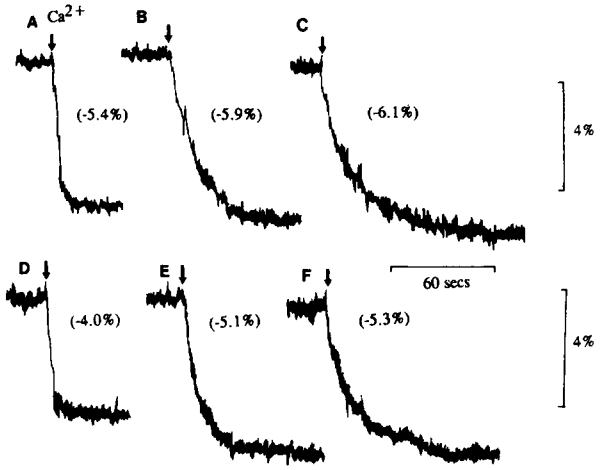

Fig. 3. The effects of bis-phenol on the fluorescence of FITC-labelled SR. 10 μg of FITC-labelled SR were incubated at pH 6 (A,B,C) or pH 7 (D,E,F) at 25°C with bis-phenol. The fluorescence decrease was measured upon the addition of 0.4 mM Ca^{2+}. (A,D) were in the absence of bis-phenol, (B) in the presence of 2 μM bis-phenol, (C,E) in the presence of 3.5 μM bis-phenol and (f) in the presence of 10 μM bis-phenol. The numbers in the brackets are percentage fluorescence decrease.

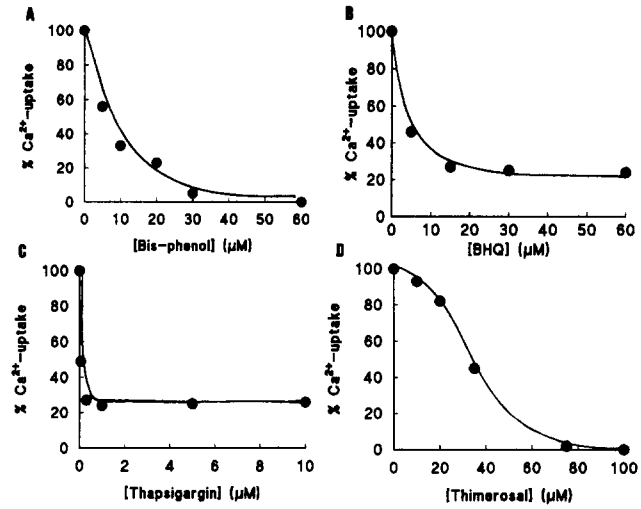

Fig. 5. The effects of bis-phenol, BHQ, thapsigargin and thimerosal on ATP-dependent Ca^{2+} uptake in cerebellar microsomes. Rat cerebellar microsomes (0.15 mg in 2 ml) were preincubated for 10 min with: (A) bis-phenol, (B) BHQ, (C) thapsigargin and (D) thimerosal. Ca^{2+} uptake was measured at pH 7.2, 37°C, initiated by the addition of 1.5 mM Mg-ATP. The data represent the means of two or more measurements with the deviation being less than 10% of the mean value.

bis-phenol over a similar concentration range and has a similar IC_{50} (9 μM). Fig. 5 also compares the effects of bis-phenol on the inhibition of Ca^{2+} uptake by cerebellar microsomes with the effects of BHQ, thapsigargin and thimerosal. As shown, although bis-phenol and thimerosal completely inhibit Ca^{2+} uptake, thapsigargin and BHQ only inhibit Ca^{2+} uptake by approx. 70–80%. This partial inhibition by thapsigargin and BHQ has already been demonstrated in a variety of cell types [4–6]. This effect could not be accounted for by the presence of a small amount of plasma membrane sealed vesicles containing plasma membrane Ca^{2+}-ATPases, since the inclusion of 3 μM vanadate (which would completely inhibit plasma membrane Ca^{2+}-ATPases [34]) had no effect on the residual Ca^{2+} uptake observed with thapsigargin or BHQ (data not shown).

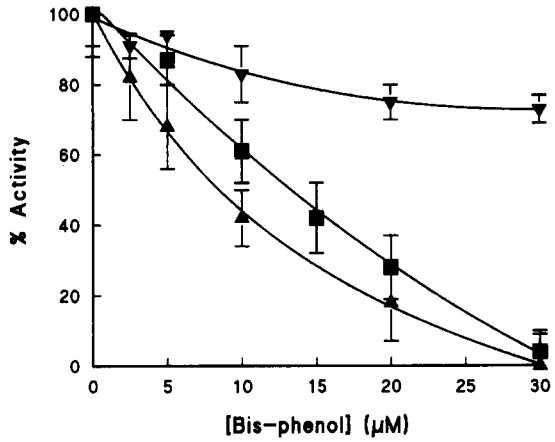

Fig. 4. The effects of bis-phenol on the activities of other ATPases. The membranes were preincubated with 0–30 μM bis-phenol for 10 min prior to the rates of ATP hydrolysis being measured at pH 7.2, 37°C. (■) Rat cerebellar ER Ca^{2+}-ATPase, (▲) human red blood cell plasma membrane Ca^{2+}-ATPase and (▼) rat kidney medulla Na^+/K^+-ATPase. 100% activity of the: ER Ca^{2+}-ATPase corresponds to 0.18 I.U./mg membranes; plasma membrane Ca^{2+}-ATPase corresponds to $1.24 \cdot 10^{-5}$ I.U./mg membranes and Na^+/K^+-ATPase corresponds to 0.31 I.U./mg membranes. The data are presented as the means ± S.E. of 3–6 determinations.

Fig. 6A shows the effects of bis-phenol on fura-2 loaded HL-60 cells. In the absence of external free Ca^{2+}, the intracellular Ca^{2+} concentration was estimated to be 70 nM. Upon addition of bis-phenol, and after a delay of approximately 150 s, a transient rise in intracellular Ca^{2+} concentration was observed, which could only be derived from intracellular Ca^{2+} stores. ATP is a known agonist of HL-60 cells which is able to mobilise calcium via the inositol phosphate pathway [26]. However, once these cells have been treated with bis-phenol they are resistant to Ca^{2+} mobilisation by ATP. Fig. 6B shows the dose-dependent effects of bis-phenol on intracellular [Ca^{2+}] in HL-60 cells in the presence and absence of external calcium. The higher increase in intracellular Ca^{2+} in the presence of external Ca^{2+} is due, at least in part, to inhibition of the plasma membrane Ca^{2+}-ATPases.

4. Discussion

Sokolove et al. [27] showed that a number of phenolic antioxidants including nonylphenol, butylated hydroxytoluene (BHT) and bis-phenol are potent inhibitors of skeletal muscle Ca^{2+}-ATPase, but do not greatly affect other ATPases such as the H^+-ATPase and from our study

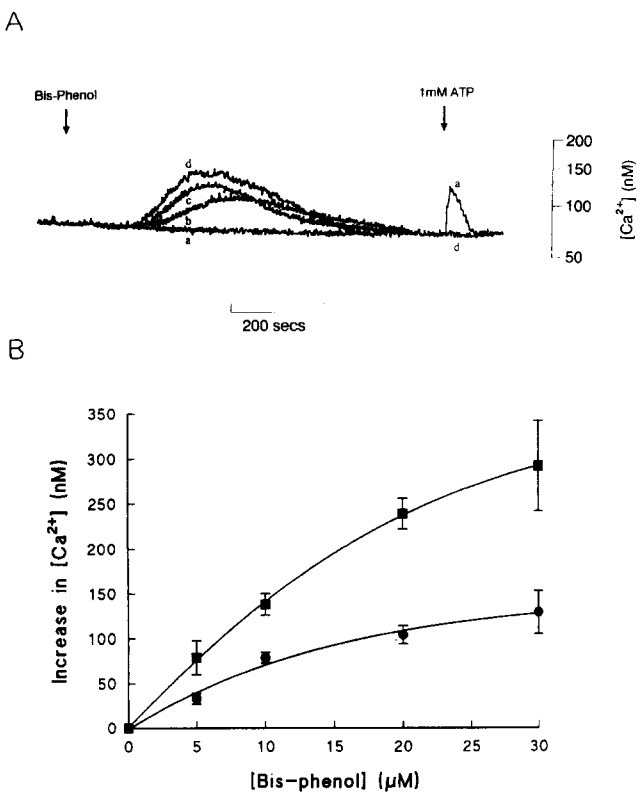

Fig. 6. The effects of bis-phenol and ATP on Ca^{2+} mobilization in HL-60 cells. (A) Bis-phenol or ATP were added to fura-2 loaded HL-60 cells (0.5 million/ml) in Hanks balanced salt medium containing 5 mM EGTA. Trace (A) shows the steady-state level of fura-2 fluorescence, calculated as free Ca^{2+} concentration, of HL-60 cells and the calcium mobilization caused by 1 mM ATP. Traces (B–D) show the effects of addition of 5, 10 and 20 μM bis-phenol, respectively. Trace (D) also shows the effects of the addition of 1 mM ATP after Ca^{2+} mobilization with 20 μM bis-phenol. (B) The effects of bis-phenol (0–30 μM) on fura-2 loaded HL-60 cells in the presence of 1.3 mM external Ca^{2+} (■) and in the absence of external Ca^{2+} (●). The data are presented as the means ± S.E. of 3–5 determinations.

the Na^+/K^+-ATPase. More recently, BHQ has also been demonstrated to be an inhibitor of muscle and non-muscle of Ca^{2+}-ATPases [2,6,9] and has been shown to induce Ca^{2+} release from intact cells [28]. Inhibitors such as BHQ and thapsigargin have been used extensively to define selective Ca^{2+} stores within cells [1–6]; some of these stores have been shown to be thapsigargin- and BHQ-insensitive, as Ca^{2+} uptake could not be completely inhibited [4–6].

There are believed to be several distinct Ca^{2+}-ATPase isoforms in non-muscle tissues as determined by genetic [11–13] and immunological analyses [14,15,29]. Although these isoforms have similar kinetic properties to each other and to the muscle isoforms of the Ca^{2+}-ATPase [18,30], they may have different sensitivities to inhibitors. It is known that some inhibitors of the Ca^{2+}-ATPase such as thimerosal [31] (and see Fig. 5D) and orthovanadate [4,18] can completely inhibit ATPase activity and Ca^{2+} uptake in cerebellar microsomes and other tissues, but their use with intact cells is limited by their membrane impermeability. Other inhibitors such as thapsigargin and BHQ are more suitable for studies of intact cells, but result in only partial (70–80%) inhibition of Ca^{2+} accumulation [4–6] (see Fig. 5B,C). It has recently been demonstrated that two isoforms of the ATPase exist in a variety of cells, including blood platelets, with one of the isoforms being resistant to the effects of thapsigargin and the other to BHQ [14,15]. This may well explain the partial inhibition of Ca^{2+} accumulation seen with these inhibitors in microsomes and permeabilised cells. However, here we demonstrate complete inhibition of ATP hydrolysis and Ca^{2+} uptake by the cerebellar ER Ca^{2+}-ATPase with bis-phenol even though under the same conditions we show only partial inhibition of Ca^{2+} uptake with thapsigargin and BHQ (Figs. 4,5). We therefore propose that bis-phenol is a potent inhibitor of all types of ER Ca^{2+}-ATPases expressed in cerebellar microsomes, and possibly in other non-muscle cells as well.

Bis-phenol is able to release Ca^{2+} from internal stores of intact HL-60 cells in a dose dependent manner (Fig. 6A,B). However, over the concentration range used, a delay in Ca^{2+} elevation after bis-phenol treatment was observed (Fig. 6A). The cause for this delay is uncertain, but a similar delay has also be observed with BHQ [32] and may be due to slow permeation of bis-phenol across the plasma membrane. As the addition of ATP to HL-60 cells stimulates inositol 1,4,5-triphosphate ($InsP_3$) production and hence causes Ca^{2+} mobilization from $InsP_3$-sensitive Ca^{2+} stores, the cells were challenged with ATP after bis-phenol treatment. Since no ATP-dependent Ca^{2+} mobilization was observed, bis-phenol must be releasing Ca^{2+} from stores which include those sensitive to $InsP_3$ (Fig. 6A).

Bis-phenol may prove to be useful in conjunction with other ATPase inhibitors to study intracellular Ca^{2+} stores which have previously been classified as thapsigargin- or BHQ-insensitive and which contain different types or isoforms of the Ca^{2+}-ATPase. It remains to be determined whether the different Ca^{2+} ATPases co-exist within the same or different Ca^{2+} stores in cells.

Recently, interest has focused on the detailed mechanism of inhibition by these molecules [8–10,22]. The mechanism of the Ca^{2+}-ATPase is usually discussed in terms of the E_2–E_1 model originally proposed by De Meis and Vianna [33]. This model postulates two major conformational states of the enzyme, E_1 and E_2. These two states differ in their affinity for Ca^{2+}, with the E_1 state having high-affinity (cytoplasmic facing) Ca^{2+} sites and the phosphorylated form of E_2 having low-affinity (luminal facing) Ca^{2+} sites. Following binding of Ca^{2+} and ATP to E_1, phosphorylation of the ATPase can take place to form the E_1PCa_2 intermediate. After a conformational change to E_2PCa_2, loss of Ca^{2+} ions into the lumen of the SR allows dephosphorylation of the ATPase and return to the E_1 state to repeat the cycle. Recent studies on the mechanism of action of the inhibitors nonylphenol [22], BHT

[22], thapsigargin [8,9] and BHQ [9,10] have shown several steps in the enzyme cycle to be affected. All these inhibitors have been shown to shift the E_1–E_2 equilibrium towards E_2 by reducing the rate of the E_2 to E_1 transition [8,9,22]. All except BHT have also been shown to inhibit phosphoenzyme formation by P_i [8–10,22]. Although thapsigargin dramatically decreases the ATPase affinity for Ca^{2+} [9], nonylphenol and BHQ have only small effects on Ca^{2+} affinity [9]. Although these studies show that the inhibitors do not affect just one particular step, the observed decrease in the rate of the E_2 to E_1 transition is sufficient to explain the decrease in the steady-state of ATP hydrolysis, at least for thapsigargin and BHQ [9].

As shown in Fig. 3 binding of bis-phenol to the ATPase also results in a shift of the E_2–E_1 equilibrium towards E_2 with a decrease in the rate of the E_2 to E_1 transition. By comparison with the effects of thapsigargin and BHQ, the effect of bis-phenol on the rate of the E_2 to E_1 transition is likely to account for its inhibitory effect. As shown in Fig. 2A, addition of bis-phenol has no significant effect on the Ca^{2+}-dependence of ATPase activity. Although a shift in the E_2–E_1 equilibrium towards E_2 would be expected to reduce the affinity of the ATPase for Ca^{2+}, a shift in the Ca^{2+}-dependence of ATPase activity (apparent Ca^{2+} affinities) need not be observed, since effects of inhibitor will be opposed by strong binding of MgATP to the E_1 conformation of the ATPase, as observed for nonylphenol [22]. This detailed study of both nonylphenol and BHT showed that the major inhibitory effect on the ATPase was at the E_2–E_1 transition, however, neither caused any changes in apparent Ca^{2+} affinities as determined by the Ca^{2+}-dependence of ATPase activity [22]. In addition the inhibition of ATPase activity by nonylphenol was more marked at low concentrations of ATP than at high [22], whereas with bis-phenol the opposite effect is observed (Fig. 2B). Indeed, in the presence of bis-phenol, plots of ATPase activity as a function of ATP concentration fit to a simple Michaelis-Menten equation, suggesting abolition of the stimulatory effect of high concentrations of ATP observed in the absence of bis-phenol (Fig. 2B). Such an effect of bis-phenol could follow if the E_2 to E_1 step was rate controlling at high concentration of ATP but not at low concentrations; it is known that binding of ATP increases the rates of a number of the steps in the reaction sequence, including that of dephosphorylation, which is rate controlling under some conditions (see Ref. [17]). Alternatively, bis-phenol could reduce the affinity of the regulatory site on the ATPase for ATP.

In conclusion we have demonstrated that bis-phenol is a potent inhibitor of the SR, ER and plasma membrane Ca^{2+}-ATPases, shifting the E_1–E_2 equilibrium towards E_2 and slowing down the E_2 to E_1 transition. Bis-phenol may prove useful in studying intracellular Ca^{2+} stores since it is able to mobilise calcium from all stores which contain Ca^{2+}-ATPases including those which are thapsigargin- and BHQ-insensitive and $InsP_3$-sensitive.

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