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THE WIDELY UTILIZED BROMINATED FLAME RETARDANT, TETRABROMOBISPHENOL A (TBBPA) IS A POTENT INHIBITOR OF THE SERCA Ca^{2+} PUMP.

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Short title: The effects of TBBPA on the SERCA Ca^{2+} pump

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SUMMARY

Tetrabromobisphenol A (TBBPA) is currently the most widely used type of brominated flame retardant (BFR), employed to reduce the combustibility of a large variety of electronic and other manufactured products. Recent studies have indicated that BFRs, including TBBPA, are bio-accumulating within humans at relatively high concentrations. BFRs, including TBBPA have also been shown to be cytotoxic and potentially endocrine disrupting in a variety of cell types. Furthermore, TBBPA has specifically been shown to cause disruption of Ca^{2+} homeostasis within cells, which may be the underlying cause of its toxicity. In this study, we have demonstrated that TBBPA is a potent non-isoform specific inhibitor of the SERCA Ca^{2+}-ATPase (apparent Ki 0.46 – 2.3μM), thus, we propose that TBBPA inhibition of Ca^{2+} ATPase contributes, at least in part, to Ca^{2+} signalling disruption. TBBPA binds directly to the ATPase without the need to partition into the phospholipid bilayer. From activity data and Ca^{2+}-induced conformational data, it appears that the major effect of TBBPA is to decrease the Ca^{2+} ATPase affinity for Ca^{2+} binding (increasing the K_d from approx. 1μM to 30μM in the presence of 10μM TBBPA). Low concentrations of TBBPA can quench the tryptophan fluorescence of the ATPase and this quenching can be reversed by 2,5-di-tert-butylhydroquinone (BHQ) and 4-n-nonylphenol, but not thapsigargin, indicating that TBBPA and BHQ may be binding to similar regions in the Ca^{2+}-ATPase.

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INTRODUCTION

Ca^{2+} plays an important role in many cellular signalling processes. Indeed, inadequate or prolonged elevations of intracellular [Ca^{2+}] levels may lead to deleterious effects, ranging from failure in signal transduction to apoptotic processes within and between cells [1]. Therefore, Ca^{2+} homeostasis in the cytosol is very crucial to cell viability and function, with the potential for serious consequences arising when normal control is disturbed. The sarco/endoplasmic reticulum Ca^{2+}- ATPase (SERCA) has long been implicated as playing a central role in the mechanism of Ca^{2+} transport across the membrane, from the cell cytosol into the endoplasmic reticulum and this is one of the major mechanisms by which low levels of free cytosolic Ca^{2+} concentration are maintained in the cell. SERCA Ca^{2+} pumps belong to the P- type family of ion transporters [2] and three isoforms of this pump is known to exist which have a tissue-specific distribution [3].

The activities of SERCA Ca^{2+} pumps have been found to be altered by a wide spectrum of lipophilic molecules, including a number of environmental endocrine disrupters [4-6]. While the majority of these molecules have non-specific mechanistic effects on these pumps, some of these hydrophobic molecules interact specifically at distinct sites on the protein and in some cases these molecules have proved invaluable in elucidating mechanistic steps within the Ca^{2+} transport processes [7-10].

Tetrabromobisphenol A (TBBPA) is a highly lipophilic halogenated aromatic molecule and currently the most widely used type of brominated flame retardant (BFR) [11]. TBBPA and other BFRs are employed as additives in the manufacture of printed circuit boards and polymers used to make electrical equipment, textiles, adhesives, foam furniture and insulating foams, in order to reduce the risk of combustion [11].

Due to their wide spread use and abundance, recent studies have shown that BFRs exist in the environment far from their location of production and / or use; and that the concentrations of some of the BFRs, both in the environment and in humans, are rapidly increasing [12]. Concentrations of various BFRs have been found at levels as high as 1 mg/ kg of dried sewage slurry [13] and up to 160 ng/g in some samples of human serum lipid (equivalent to ≈ 0.3μM) [14]. More specifically, TBBPA was found to be as high as 3.8 ng/ g lipid in serum samples from electronics workers [15].

In-vitro studies have demonstrated that TBBPA is toxic to a variety of mammalian cell lines [16], as well as primary cells including; hepatocytes [17], splenocytes [18] and cerebella granule cells [19, 20]. In addition, TBBPA also shows estrogenic activity since it enhances the estrogen-dependent proliferation of MtT/E-2 cells [21]. Furthermore, TBBPA at low μM concentrations has been shown to modulate a number of cell signalling processes such as the activities of tyrosine kinases, MAP kinases, protein kinase C and also elevate levels of reactive oxygen species and cytosolic Ca^{2+}, when

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added to neutrophils [22]. A recent study has also shown that low concentrations of TBBPA can also increase cytosolic Ca^{2+} levels in cerebellar granule cells leading to cell death [20].

The widespread production and use of BFRs and the strong evidence of increased contamination of the environment and humans by these chemicals has led us to investigate the effects of TBBPA; the most commonly used BFR, on SERCA Ca^{2+} pumps as a mechanism of disrupting Ca^{2+} signalling pathways.

MATERIALS AND METHODS

Tetrabromobisphenol A was purchased from Acrōs Organics (purity given as 97%). All other reagents were of analytical grade. TBBPA was dissolved in dimethylsulfoxide to give a stock solution of 20mM and the solvent was never more than 1.0% (v/ v) in the assays performed. The pKa of TBBPA was determined in aqueous solution by the method described in Bilmen et al, (2003) [8].

Native sarcoplasmic reticulum (SR) was prepared from rabbit skeletal muscle as described by Michelangeli & Munkonge (1991) [23] and typically had a purity of ≈80% Ca^{2+}-ATPase. Porcine cerebella microsomes were prepared as described in [24].

Ca^{2+}-ATPase Activity

Ca^{2+}-ATPase activity in cerebella microsomes was performed employing the phosphate liberation assay as described by Longland et al. (1998) [25].

The effects of TBBPA on SR Ca^{2+}-ATPase activity was investigated as a function of [Ca^{2+}], [ATP] and at different pH values, employing a coupled enzyme assay as previously described in [23, 25]. Typically, between 11-22 µg/ml of ATPase protein was added to a buffer containing 40mM Hepes/KOH (pH 7.2 or pH 6.0), 5mM $MgSO_4$, 0.42mM phosphoenolpyruvate, 0.15mM NADH, 8.0 U pyruvate kinase, 20U lactate dehydrogenase, 1.01mM EGTA and 2.1mM ATP. In all experiments, unless otherwise stated, a free [Ca^{2+}] of 6µM (pCa 5.2) was used. Free Ca^{2+} concentrations were calculated as described in [26].

Phosphorylation studies

Phosphorylation of ATPase by [γ-^{32}P]ATP was carried out at 25°C as described by Michelangeli et al., 1990 [4,7,8]. ATPase was diluted to 0.1 mg/ ml in 20mM Hepes/Tris (pH 7.2) containing 100mM KCl, 5mM $MgSO_4$ and 1mM $CaCl_2$ in a total volume of 1ml. 0.5 and 5mM stocks of ATP with specific activities of 100 or 10Ci/ mol, respectively were made in the above buffer, to cover the range

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between 0 and 25μM. Reaction was started by the addition of [γ-^{32}P]ATP and stopped by the addition of ice-cold 40% (w/ v) TCA after 15secs. The assay was then placed on ice for about 30mins prior to filtration through Whatman GF/C filters. The filters were washed with 30ml of 12% (w/ v) TCA/ 0.2M H_3PO_4 and left to dry. The filters were placed in scintillant and counted.

TBBPA binding to SERCA

The binding of TBBPA to SERCA was measured by monitoring its ability to quench the tryptophan fluorescence of the Ca^{2+}-ATPase. The protein was diluted to 1 μM in a buffer containing 40mM Hepes/KOH (pH 7.2 or 6.0), 1mM EGTA and 100mM NaCl. Fluorescence intensity was measured in a Perkin-Elmer LS50B Fluorescence Spectrophotometer at 25°C (excitation 280nm, emission 340nm). In other experiments, some inhibitors of Ca^{2+}-ATPase {i.e. Thapsigargin (TG), 2,5-di-tert-butylhydroquinone (BHQ) and Nonylphenol (NP)} were added separately after 1 μM TBBPA, in order to assess whether they could displace TBBPA binding and therefore reverse TBBPA-induced fluorescence quenching.

The rate constants for TBBPA binding to the Ca^{2+}-ATPase and to phospholipid bilayers were assessed by monitoring either the rate of tryptophan fluorescence quenching of the Ca^{2+} ATPase or of the membrane probe, pyrene dodecanonic (PDA) incorporated into phospholipid bilayers [27]. Rapid kinetic fluorescence measurements of TBBPA binding to the Ca^{2+} ATPase was performed using a stopped-flow Fluorescence spectrophotometer (Applied Photophysics, model SX17 MV). Briefly, the sample handling unit possesses two syringes, A and B (drive ratio 10:1), which are driven by a pneumatic ram. Tryptophan fluorescence quenching of the protein was monitored at 25°C when 1μM Ca^{2+}-ATPase (in 20mM Hepes, 100mM KCl, 5mM $MgSO_4$, pH 7.2) was rapidly mixed with 10μM (final concentrations) of TBBPA in DMSO, exciting at 280 nm and measuring the emission above 320 nm using a cut-off filter. In order to assess the binding of TBBPA to phospholipid membranes, unilammellar liposomes of dioleylphosphatidylcholine containing PDA at a molar ratio of 500: 1 were prepared in the hepes buffer as above, followed by probe sonication and a freeze-thaw cycle. The time course of TBBPA quenching of PDA within DOPC liposomes was measured using a Hi-Tech SFA-12 stopped-flow accessory attached to a Perkin-Elmer LS50B fluorimeter by mixing 90 μM liposomes (equivalent concentration of lipids found associated with 1μM SR Ca^{2+} ATPase within the SR vesicles) with 10 μM TBBPA. Excitation was at 342 nm and emission at 395 nm.

Intrinsic tryptophan fluorescence measurements to monitor Ca^{2+}-induced conformational changes of Ca^{2+}ATPase

The conformational change induced by the addition of Ca^{2+} to the ATPase was monitored by observing the change in intrinsic tryptophan fluorescence [as described in [4,7,8]. 50μg/ml ATPase (in the absence and presence of 3.5μM TBBPA) was added to a buffer containing 20mM Hepes/Tris, 100mM $MgSO_4$, (pH 7.0) and the induced-conformational change was measured as a percentage

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change in total tryptophan fluorescence, over a range of free [Ca^{2+}] (10 - 100µM), was measured at 25°C (excitation 285nm, emission 325nm).

Measurement of the transient kinetics of the conformational changes associated with Ca^{2+}-binding and dissociation

Rapid kinetic fluorescence measurements were performed using a stopped-flow Fluorescence spectrophotometer as described in [7,8]. In the Ca^{2+} binding experiments, syringe A, containing the Ca^{2+}-ATPase (1µM) in 20mM Hepes/Tris (pH7.2), 100mM KCl, 5mM $MgSO_4$, 50µM EGTA was rapidly mixed with syringe B, containing 1mM Ca^{2+} (final concentration). In Ca^{2+} disscociation experiments, syringe A, containing the Ca^{2+}ATPase (1µM) in 20mM Hepes/Tris (pH7.2), 100mM KCl, 5mM $MgSO_4$, 100µM Ca^{2+} was rapidly mixed with syringe B, containing 2mM EGTA (final concentration). Tryptophan fluorescence was monitored at 25°C by exciting the Ca^{2+}-ATPase (rapidly mixed with buffers containing either Ca^{2+} or EGTA) at 280nm and measuring the emission above 320nm using a cut off filter.

Molecular Modelling

The program Molegro Virtual Docker 2007 (with the standard default settings) was used in order to predict the ligand–protein interactions of the energy minimised conformational structure of TBBPA to the Ca^{2+}-ATPase. The atomic coordinates for the E2 (TG + BHQ) form of the Ca^{2+}-ATPase (PDB code 2AGV) was used for this virtual docking procedure. Figures were produced using the Swiss-pdbViewer.

RESULTS

Fig.1 shows that TBBPA is a potent inhibitor of the skeletal muscle SERCA 1a isoform of the Ca^{2+}-ATPase and that this inhibition is pH sensitive. At pH 7.2 the apparent inhibition constant (app. Ki: concentration causing 50% inhibition under these experimental conditions) was determined to be 1.7±0.3µM, while at pH 6.0 the potency increased significantly (app. Ki, 0.46±0.05µM). As low pH favours the E2 state of the Ca^{2+}ATPase [28], this might indicate that TBBPA preferentially binds to and stabilizes the ATPase in this conformation. This pH effects is, however, unlikely to be due to protonation of the phenol groups on TBBPA since the pKa for TBBPA was determined to be 9.8±0.3, (typical of other phenol groups) and which would indicate that TBBPA is essentially fully protonated at both pH6 and pH 7.2. Cerebellum is known to express both SERCA2b and SERCA3. Fig. 1 (inset) shows that TBBPA can also inhibit the non-striated muscle SERCA isoforms SERCA2b and SERCA3, since it inhibits the cerebellar microsomal Ca^{2+}-dependent ATPase activity with an app. Ki of 2.33±0.45µM at pH 7.2 (i.e. similar to that determined for SERCA 1a). Therefore it would seem

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that TBBPA is a non-selective SERCA isoform inhibitor, unlike some other inhibitors previously examined [9]. Complete inhibition, in all cases, was observed at or below 10μM TBBPA. In comparison, we have also shown that the related, non-brominated, compound; bisphenol A, a known endocrine disrupter which is used as a plasticising agent, is a very much weaker inhibitor of the SR Ca^{2+} ATPase with a Ki of 233±30 μM at pH 7.2 [6,29].

Fig. 2A shows the effect of TBBPA on ATPase activity as a function of free [Ca^{2+}]. Ca^{2+}-ATPase activity exhibited a classical 'bell-shaped' profile with respect to free [Ca^{2+}] in the absence and presence of TBBPA. The stimulatory phase is associated with Ca^{2+} binding to the ATPase in an E1 (high affinity) form, increasing turnover rate, while the inhibitory phase, is associated, in part, with the binding of Ca^{2+} to the E2 (low affinity) form of the enzyme. In the absence of TBBPA, the results gave V_{max} of 5.0±0.2 I.U/mg, with a K_m value of 0.62±0.06μM for the stimulatory phase and a K_m value of 0.62±0.06mM for the inhibitory phase. Whereas, in presence of TBBPA (between 1-4 μM), the data showed both a decrease in the maximal activity and an increase in the K_m for the stimulatory phase. At 1 μM TBBPA, the V_{max} was slightly decreased to 4.81±0.40 I.U/mg, while the K_m's for the stimulatory and inhibitory phases increased to 1.04±0.1μM and 0.68±0.07mM, respectively. The changes became more apparent at 2.0μM TBBPA, as the V_{max} decreased to 2.94±0.15 I.U/mg, while the K_m's increased further to 1.96±0.15μM and >1mM for the stimulatory and inhibitory phases, respectively. Furthermore, in the presence of 4μM TBBPA, V_{max} decreased to 3.33±0.25 I.U/mg while both K_m's increased significantly (11.0±0.10μM, for the stimulatory and >1mM, for the inhibitory). These results suggest that TBBPA may be decreasing the binding affinity of Ca^{2+}-ATPase for Ca^{2+}.

Fig. 2B shows the activity of Ca^{2+}-ATPase at various [ATP] in the absence and presence of 1.5μM TBBPA. The complex biphasic profiles have previously been modeled assuming a bi-Michaelis-Menton process described in [5,8,30], where it is assumed that, two independent ATP binding events occur during enzymatic turnover, designated as the high affinity catalytic site and the lower affinity regulatory site. ATP binds and phosphorylates the ATPase at the high affinity catalytic site while, the low affinity regulatory site accelerate ATPase cycle rate. In the absence of TBBPA, the data could be fitted assuming a catalytic K_m and V_{max} of 0.5μM and 1.0IU/mg, respectively; and a regulatory K_m and V_{max} of 1.0mM and 1.8IU/mg, respectively. In the presence of 1.5μM TBBPA, only the catalytic V_{max} appeared substantially different compared to control, while all other parameters showed little or no differences (i.e. cat. K_m = 0.5μM, cat. V_{max} = 0.2 IU/mg; reg. K_m = 0.4mM, reg. V_{max} = 1.4IU/mg).

In order to assess the possible effect of TBBPA on the phosphorylation of the ATPase by ATP, experiments were performed at the optimal ATP concentration (25 μM) and varied concentrations of TBBPA. As shown in Fig 3A, the level of Ca^{2+}-ATPase phosphorylation was reduced in the presence

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of TBBPA with a K_i of 2.0±0.5 μM. As changes in the affinity of ATP binding to the ATPase can alter the level of phosphorylation by affecting the K_{ATP} (concentration of ATP required to achieve 50% of the E-P_{max}), the effects of ATP concentration in the absence and presence of 2.5μM TBBPA were further investigated (fig. 3B). As shown in fig 3B, E-P_{max} and K_{ATP} values of 9.9±0.9 nmol/mg and 0.9±0.5 μM, respectively were obtained for the control, whereas in the presence of 2.5μM TBBPA, the data gave E-P_{max} and K_{ATP} values of 6.7±0.6 nmol/mg and 2.3±0.8 μM, respectively. Thus, TBBPA caused a decrease in the maximum phosphorylation level of the ATPase (fig. 3A) but had only relatively modest effects on the ATP affinity (ie K_{ATP}).

Fig 4A shows the binding of TBBPA to Ca^{2+}-ATPase, monitored by tryptophan fluorescence quenching, at pH 7.2 and pH 6.0. Both sets of data could be reasonably fitted using a simple one-site binding relationship where the K_d values were determined to be 16 and 27 μM, for the pH 6 and pH 7.2 data, respectively (statistical analysis of goodness-of-fit as assessed by chi^2 tests were determined to be around 0.97, shown by the dotted lines). Slightly better fits to the data could, however, be achieved assuming a two-site binding relationship ($chi^2 \geq 0.992$). Therefore our data would favour the possibility that fluorescence quenching of the ATPase by TBBPA could be best represented assuming a high and low affinity binding sites. The solid lines presented in fig 4A for the pH 6.0 data was achieved assuming K_d values of 1.0 μM and 19 μM, while the fit to the pH 7.2 data was achieved assuming K_d values for the two sites of 2.2 μM and 44 μM, for the high and low affinity sites, respectively. These results would therefore indicate that the high affinity K_d values obtained here probably relates to the binding sites on the ATPase which are associated with inhibition, since they are comparable with the Ki values. The fluorescence quenching of the Ca^{2+}-ATPase at low concentrations of TBBPA (1μM) was used to determine whether other commonly used hydrophobic inhibitors competed for the same site on the ATPase as TBBPA. This was achieved by determining whether the quenching caused by 1μM TBBPA could be reversed by displacing it from its binding site. Fig. 4B shows fluorescence traces of both TBBPA quenching followed by the addition of a variety of inhibitors. It can be clearly seen that thapsigargin (2 x 1μM), showed no reversibility of the TBBPA quenching, however, both BHQ (2 x 5μM) and nonylphenol (2 x 5μM) inhibitors could significantly reverse TBBPA quenching upon their addition, indicating competitive binding. The displacement of TBBPA by Cyclopiazonic acid (CPA), another commonly used SERCA inhibitor was also assessed; but as CPA caused substantial tryptophan fluorescence quenching itself, its ability to displace TBBPA from the ATPase could not be determined by this method. For the sake of completeness, Fig. 4B also shows the effects of these inhibitors on the Ca^{2+}-ATPase tryptophan fluoresence in the absence of TBBPA. As can be seen these inhibitors do not themselves cause an increase in tryptophan fluorescence of the Ca^{2+}-ATPase in the absence of TBBPA, although BHQ and TG did cause a slight

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(1-2%) quenching in fluorescence.

In order to assess whether TBBPA binds directly to the Ca^{2+}-ATPase or indirectly, via the phospholipid bilayer, the rate of TBBPA binding to the Ca^{2+} ATPase and to DOPC unilammellar liposomes (at the same lipid concentration found within the SR vesicles) was investigated. Fig. 5A shows the rate of quenching of tryptophan fluorescence within the Ca^{2+} ATPase. Quenching was completed within less than 50 ms and the quenching process could be fitted to a simple first order decay process with a rate constant of $90s^{-1}$. The rate of TBBPA quenching of PDA within the DOPC membranes was considerably slower i.e. requiring about 10 s for completion (rate constant $0.24s^{-1}$; fig. 5B). These results would suggest that TBBPA binds directly to the Ca^{2+}-ATPase, without the need to partition first, into the phospholipid bilayer.

Fig 6A shows the effect of 3.5 μM TBBPA on the tryptophan fluorescence changes of Ca^{2+}-ATPase upon the addition of a range of free Ca^{2+} concentrations, which is often used to assess the affinity of Ca^{2+} binding to the ATPase [7,8,31]. In the absence of TBBPA, the addition of Ca^{2+} gave a ΔF_{max} value of 9.1±0.3% and an app. K_d of 0.7±0.1μM, whereas in the presence of 3.5μM TBBPA, there was a decrease in the maximal fluorescence change (ΔF_{max} value of 5.21±0.04%) and a substantial in increase in app. K_d. This data also suggests that TBBPA decreases the affinity of Ca^{2+}-ATPase for Ca^{2+}. Fig 6B illustrates the effect of varying [TBBPA] on tryptophan fluorescence changes of Ca^{2+}-ATPase upon the addition of Ca^{2+} at pH 7.2. In the absence of TBBPA, addition of Ca^{2+} to the ATPase caused a 9.09 +/- 0.31% decrease in tryptophan fluorescence, whereas in the presence of 10μM TBBPA, a 3.76 +/- 0.66 % decrease in tryptophan fluorescence resulted. The IC_{50} for TBBPA under these conditions is about 3.5μM. It is also apparent from the data in fig. 6A that there is also a small increase in fluorescence over the 10nM to 10μM free [Ca^{2+}] concentration range in the presence of 3.5μM TBBPA. However, since under these experimental conditions, only a proportion of the Ca^{2+}-ATPase is affect by TBBPA (fig. 6B), this would suggest that this component of the fluorescence change is due to the proportion of ATPase unaffected by TBBPA (thus having an app K_d similar to control).

At pH 7.2, the rate constants for the conformational changes associated with either Ca^{2+} binding to ATPase or its dissociation from ATPase were measured in the absence and presence of TBBPA by following the Ca^{2+}-induced changes in tryptophan fluorescence using stopped-flow spectrofluorimetry. In Fig 7A & B, all the data could be fitted to a mono-exponential equation (rate constants given in table 1) as this is the simplest relationship which gave good fits to the data obtained in these experiments. As shown in table 1, the rate constant for Ca^{2+} binding decreased whereas the rate constant for Ca^{2+} dissociation increased, with increasing [TBBPA]. Consequently, the dissociation

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constants K_d for Ca^{2+} binding (calculated from the on and off rate constants) increased proportionally with increasing [TBBPA] from 1.4µM in the absence of TBBPA to almost 30µM in the presence of 10µM TBBPA (table 1).

DISCUSSION

Brominated flame retardants are becoming prevalent throughout our environment, and many of these chemicals have been shown to bio-accumulate within humans to alarmingly high levels [8,11-13]. Some recent studies have shown that in some individuals, levels in the 1000's ng/g lipid in human breast milk have been found [32]. Toxicological studies have shown that a number of these BFRs are both neurotoxic and can act as endocrine disrupters [20, 21, 33] and several studies have shown that TBBPA can in fact disturb Ca^{2+} signalling pathways at low µM concentrations [20,22]. One obvious possibility for this is that, TBBPA can modulate the activity of Ca^{2+} transporters such as SERCA Ca^{2+} pumps.

Based on the Ca^{2+}- ATPase activities data from this study, TBBPA proved to be a very potent inhibitor of the Ca^{2+}-ATPase and affects SERCA isoforms: type 1a and the non striated muscle isoforms type 2b & 3, found in cerebellar microsomes, to similar extents. The inhibition of ATPase activity by TBBPA is also pH-sensitive, with a low pH favouring increased inhibition. The fact that under optimal conditions the Ki for inhibition is only 2-3 times higher than the molarity of the ATPase in these assays would likely indicate that the Ca^{2+}-ATPase has one (or only a few binding) site(s) for TBBPA which when occupied, inhibits activity. It is also clear that there is likely to be a number of sites of differing affinities for TBBPA on the ATPase as determined from fluorescence quenching studies, however, only occupation of the high affinity site(s) is / are required to elicit enzyme inhibition. These sites are also likely to be accessible directly from the aqueous phase, rather than via the lipid bilayer.

From the activity versus [ATP] and the phosphorylation data, it is unlikely that the major factor causing inhibition is due to TBBPA effects on ATP binding or phosphoryl- transfer. However, what does appear to be the major factor for inhibition is the observation that, TBBPA dramatically reduces the affinity of the ATPase for Ca^{2+} binding and this reduction in binding affinity is due to both a decrease in the rate of binding and an increase in the rate of Ca^{2+} dissociation from the ATPase. The calculated affinity for Ca^{2+} binding was shown to be dramatically reduced from (1.4 µM to 30 µM) in the presence of TBBPA.

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Binding site competition studies, utilizing the displacement of TBBPA (which quenches tryptophan fluorescence of the Ca^{2+}-ATPase) with a variety of other Ca^{2+}-ATPase inhibitors has shown that the quenching could be substantially reversed by BHQ and nonylphenol, both of which have been shown to stabilize the Ca^{2+} ATPase in an E2 conformational state [4, 10]. The fact that TBBPA is a more potent inhibitor at low pH (which favours the E2 form [28], and the added fact that TBBPA decreases the Ca^{2+} affinity of the ATPase (again consistent with being in an E2 state), would lead us to propose that TBBPA also inhibits the Ca^{2+} ATPase by stabilizing it in a E2 conformation. However, as TBBPA quenching of the ATPase is not reversed by thapsigargin (which again stabilizes the E2 form [10]), this would indicate that the site occupied by thapsigargin is distinct from that of TBBPA. Indeed, recent crystallographic studies have shown that thapsigargin and BHQ do bind to distinct sites within the Ca^{2+}-ATPase [34], with BHQ binding to a hydrophobic pocket of the E2 state within the transmembrane regions encompassed by M1, M2, M3 and M4. Using the high accuracy molecular docking program Molegro Virtual Docker 2007, two potential binding sites for TBBPA on the E2 form of the Ca^{2+}-ATPase were predicted (figure 8A). These two sites are distinct from the thapsigargin binding site and are both located within a large cavity encompassed by the transmembrane helices M1, M2, M3 and M4, and enclosed at the top of the pocket by regions of the phosphorylation domain. Figure 8B shows the predicted TBBPA binding site 1, which is in an identical position to where BHQ binds within the crystal structure [34], i.e. in a hydrophobic region at the bottom of the cavity encircled by; Asp 59, Leu 61 and Val 62 (within M1); Leu 253 (within M3); and Pro 308, Leu 311 and Pro 312 (within M4). This region within M4 is close to the Ca^{2+} binding site II and could well explain the effect of TBBPA on the affinity for Ca^{2+} binding. Figure 8C shows details of the predicted binding site 2 found towards the top of this cavity, consisting of the top of transmembrane helix M3 (Asp 245, Thr 247 and Gln 250); the start of the phosphorylation domain at Thr 341 and Pro337 (within helix P1), and at Glu 715 and Glu 732 also within the phosphorylation domain (at the ends of strands P6 and P7, respectively). Binding at this site may account, in part, for the effect of TBBPA reducing ATP-dependent phosphorylation. Our experimental data probably favours site 1, because BHQ can reverse TBBPA quenching, however they are not necessarily mutually exclusive, as TBBPA may conceivably bind to both sites. Although there are no tryptophan residues close to these binding sites, it seems clear that since TBBPA can bind directly to the Ca^{2+}-ATPase from the aqueous phase, it most likely enters into this cavity by passing close to the Trp 107 (at the top of M2). As binding of TBBPA to the ATPase is dynamic, at any given time within some ATPases, TBBPA must therefore be in close proximity to Trp 107, for fluorescence quenching to take place.

The fact that this cavity has also been implicated as being a Ca^{2+} entry pathway, allowing Ca^{2+} to enter into specific binding sites buried deep within the transmembrane region [35], is also supported by our data that shows that TBBPA reduces Ca^{2+} binding to the ATPase. It is, however, interesting to note that from activity, mutagenesis and modelling data obtained for 2-aminoethoxydiphenyl borate

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(2-APB) (a low affinity SERCA inhibitor) which also reduced Ca^{2+} binding by affecting both Ca^{2+} association and dissociation [8]. We deduced from that study that it bound on the adjacent side of the transmembrane bundle of the SERCA protein within a hydrophobic pocket defined by M3, M5 and M6 [8], and also occluded the Ca^{2+} entry pathway. Now from this study with TBBPA, we suggest that the cavity encompassed by M1, M2, M3 and M4 forms the pathway for Ca^{2+} entry, since it also reduces the affinity for Ca^{2+} binding by affecting both the on and off rates for Ca^{2+} binding. As we know that there are two Ca^{2+} binding sites on SERCA and that a number of potential Ca^{2+} entry pathway sites have been proposed by several groups [36-40] as to the exact route for Ca^{2+} to enter and bind, it would be tempting to speculate that each of the Ca^{2+}, enters their respective binding sites via a distinct pathway, located on adjacent sides of the transmembrane helix bundle. One piece of evidence which may support this view is the fact that when the Ca^{2+} ATPase is reconstituted with short chain length phospholipids it reduces the stochiometry for Ca^{2+} binding from 2 to 1 without significantly affecting its affinity or the phosphorylation level [41]. Thus this indicates that the 2 Ca^{2+} binding events can, under some circumstances, be made distinct.

In summary, the most commonly used BFR, TBBPA, which has been shown to be highly cytotoxic and cause disturbances of intracellular Ca^{2+} homeostasis within cells, may elicit its effects, at least in part, by inhibiting the SERCA Ca^{2+} pumps.

Acknowledgements: Dr Jon Ride is thanked for helping with the Molegro virtual docking program. We would like to thank Houdini Ho Tin Wu for preliminary experiments. The college of Medicine, University of Ibadan, Nigeria and the School of Biosciences, University of Birmingham, UK are thanked for partial scholarship support to O.A.O. A grant from the Medical Faculty of the University of Birmingham was also awarded to undertake this work.

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Figure 1: The effects of TBBPA on Ca^{2+}-ATPase activity.

Ca^{2+}-ATPase activity at a range of TBBPA concentrations was measured in rabbit skeletal muscle SR membranes at 25°C in a buffer at pH 7.2 (■) or pH 6.0 (▲). Inset shows the effects of TBBPA on the Ca^{2+} -ATPase activity in cerebellar microsomes measured at pH7.2 and 37°C. The data points represent the mean ± SD of between 3-6 determinations

Figure 2: The effects of TBBPA on ATPase activity as a function of [Ca^{2+}] and [ATP].

(A) The effects of varying [TBBPA] and free [Ca^{2+}] on SR Ca^{2+}-ATPase activity measured at pH7.2, 25°C. The concentrations of TBBPA used were; No TBBPA (■), 1μM (▲), 2μM (▼) and 4μM (♦). (B) The effects of varying [ATP] on Ca^{2+} ATPase activity measured at pH7.2 and 25°C, in the presence of no TBBPA (■) and 1.5μM TBBPA (▲). The lines represent the best fits to the data assuming a bi-Michaelis-Menton equation, with the parameters given in the text. The data points represent the mean ± SD of between 3-6 determinations.

Figure 3: Effects of TBBPA on phosphorylation of the Ca^{2+}-ATPase by ATP.

(A) Shows the effects of TBBPA (0-20μM) on the phosphorylation of the Ca^{2+}-ATPase in the presence of Ca^{2+} (1mM) by [^{32}P]ATP (25μM), at pH7.2 and 25°C. (B) shows how ATP-dependent phosphorylation of the Ca^{2+}-ATPase over a range of [^{32}P] ATP concentrations (0- 25μM) is affected in the absence (■) and presence (▲) of 2.5μM TBBPA. The data represent the mean ± SD of 3 determinations.

Figure 4: The quenching effects of TBBPA on tryptophan fluorescence of the Ca^{2+}-ATPase.

(A) Shows the effects of TBBPA on tryptophan fluorescence of the Ca^{2+}-ATPase measured at pH 7.2 (■) and pH6.0 (▲), at 25°C. The solid lines represent the best fit to the data assuming two-site binding (parameters given in text), while the dotted lines represent the best fits to the data assuming one-site binding (parameters given in text). The data points represent the mean values ± SD of 3 determinations. (B) Shows traces of tryptophan fluorescence levels being quenched by the addition of 1μM TBBPA. Following this, two additions of thapsigargin (1μM each) were made. In subsequent experiments, the quenching of 1μM TBBPA was reversed by either two additions of BHQ (5μM each), or of nonylphenol (5μM each). Control traces in which TG, (1μM), BHQ (5μM) and NP (5μM) were added in the absence of TBBPA are also shown. Each trace is representative of 3 identical experiments.

Figure 5: Kinetics of TBBPA binding to the Ca^{2+}-ATPase and to phospholipid bilayers.

(A) Shows the rate at which the tryptophan fluorescence of the Ca^{2+}-ATPase (1μM, final concentration, which is equivalent to 90 μM phospholipid) is quenched by the addition of TBBPA

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(10μM, final concentration), at pH 7.2, 25°C. The solid line represents the best fit to the experimental data assuming a 1st order decay process (k_{obs} = 90s^{-1}). The data points represent the mean of 8 accumulated runs. (B) Shows the rate of TBBPA (10 μM) quenching of the membrane bound PDA probe within DOPC unilamellar liposomes (90μM, final concentration). The fitted line represents the best fit to the experimental data, assuming a 1st order decay process (k_{obs}= 0.24s^{-1}).

Figure 6: The effects of TBBPA on the Ca^{2+}-induced tryptophan fluorescence change of the Ca^{2+}-ATPase.

(A) Shows changes in the Ca^{2+}-ATPase tryptophan fluorescence induced by variations in free [Ca^{2+}] in the absence (▲) or presence (■) of 3.5μM TBBPA, measured at pH 7.0, 25°C. (B) Shows the effect of varied [TBBPA] on the maximal tryptophan fluorescence change induced by the addition of 100μM free [Ca^{2+}]. The data points are the mean ± SD of 3 or more determinations.

Figure 7: The effects of TBBPA on the kinetics of the Ca^{2+}-induced tryptophan fluorescence change of the Ca^{2+}-ATPase.

(A) shows the effects of various TBBPA concentrations (traces from top to bottom of 0, 2, 4 and 10μM) on Ca^{2+} binding conformational change monitored by following the increase in tryptophan fluorescence of the Ca^{2+}-ATPase upon addition of Ca^{2+}. (B) Shows the effects of various concentrations of TBBPA (from bottom to top of 0, 2, 4 and 10μM) on the conformational change following Ca^{2+} dissociation from the Ca^{2+}-ATPase, monitored as a decrease in tryptophan fluorescence. The traces are the means of 8-10 accumulated runs and the solid lines are the best fits to the experimental data assuming a 1st order process, with the parameters given in table 1. The wavy appearances of some of the traces are due to the over-sampling function on the stopped-flow, being employed to reduce the signal-to-noise levels.

Figure 8: Predicted binding sites for TBBPA.

Molegro Virtual Docker 2007 was used to predict potential TBBPA binding sites on the E2 (TG + BHQ) SERCA1a crystal structure (PBD code 2AGV). (A) shows the two potential TBBPA binding sites (labelled site 1 and site 2). In addition, the thapsigargin binding site is also shown. The transmembrane helices are colour code accordingly: M1 (red), M2 (orange), M3 (yellow), M4 (green), M5 (cyan), M6 (blue), M7 (purple), M8 (pink), M9 (dark grey), M10 (white). (B) shows a close up of TBBPA predicted binding site 1, specifically highlighting amino acids within 4Å of TBBPA and therefore able to potentially form either hydrogen bonds or Van der Waals interactions. (C) shows a close up of TBBPA predicted binding site 2.

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Table1: The kinetic parameters of Ca^{2+} binding and dissociation from the Ca^{2+}-ATPase in the presences of TBBPA

[TBBPA]μM	k_{on}($\mu M^{-1}s^{-1}$)	k_{off}(s^{-1})	K_d (k_{off}/ k_{on}) (μM)
0	10.2±0.1	14.5±0.4	1.4
2	3.4±0.1	20.4±0.7	6.0
4	2.2±0.1	25.5±1.0	11.6
10	1.8±0.1	53.6±3.4	29.8

These values are presented as means± SEM. Data presented is a result of an average of 8 – 10 individual experiments.

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FIG 1

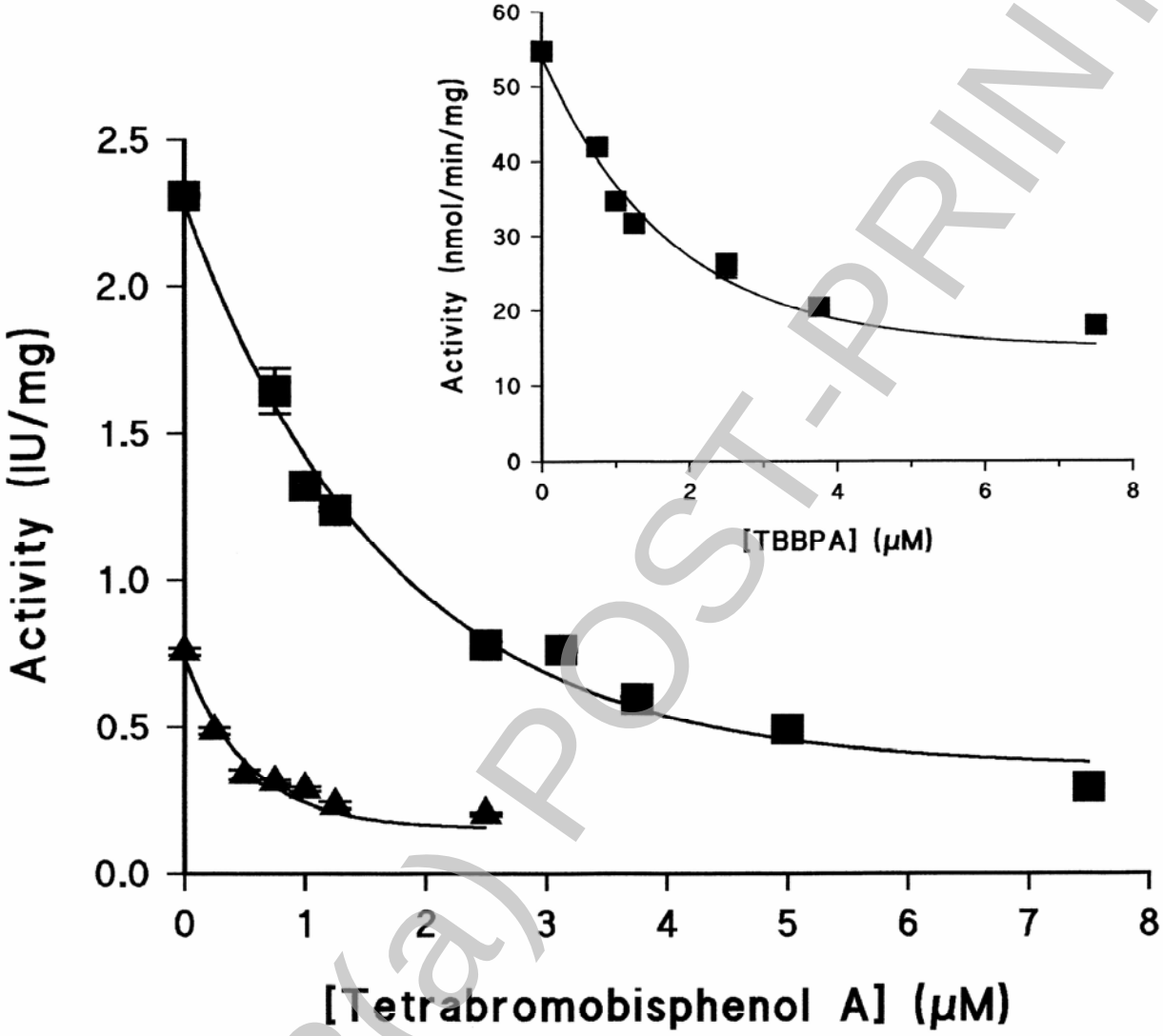

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FIG 2

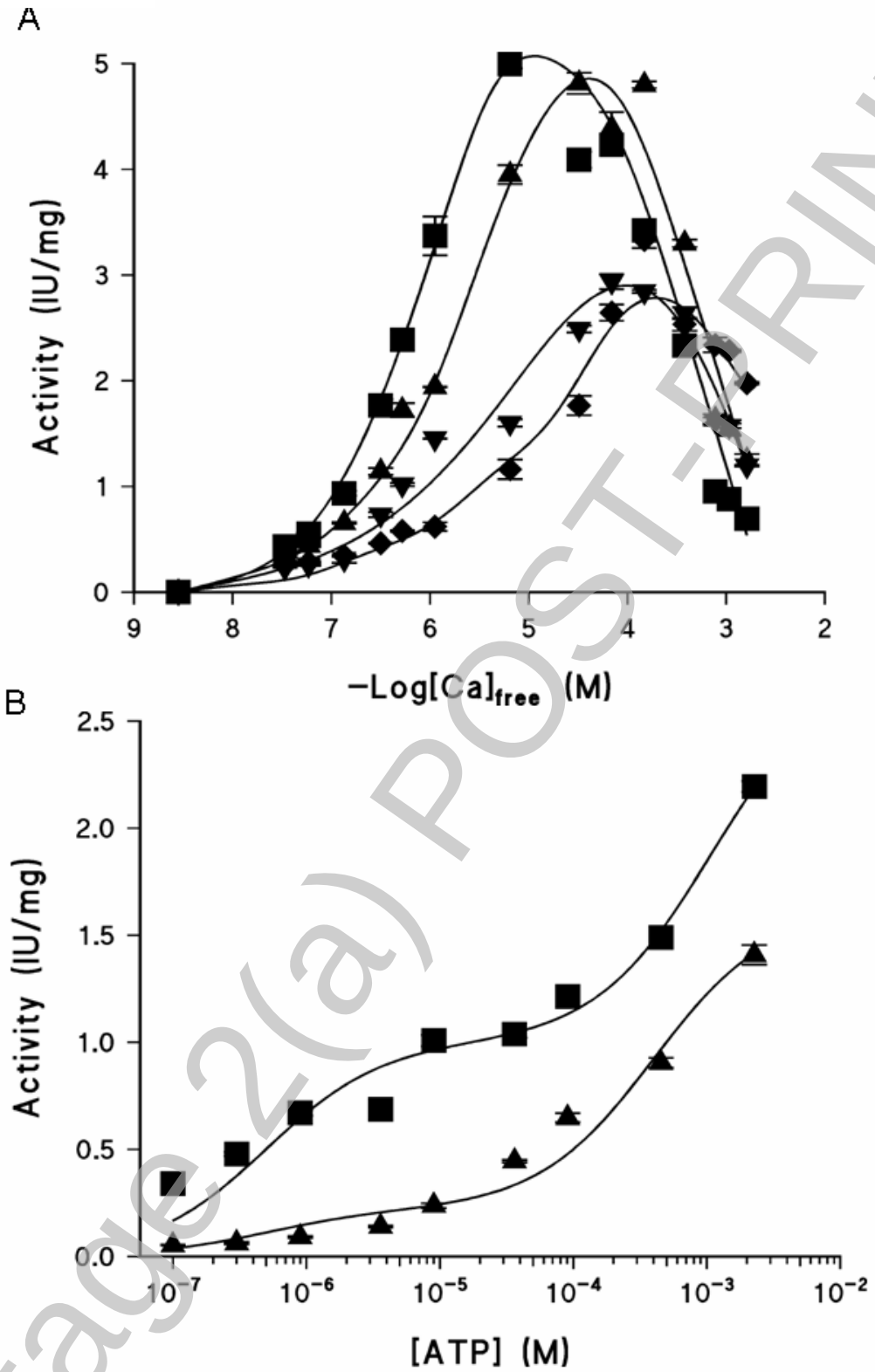

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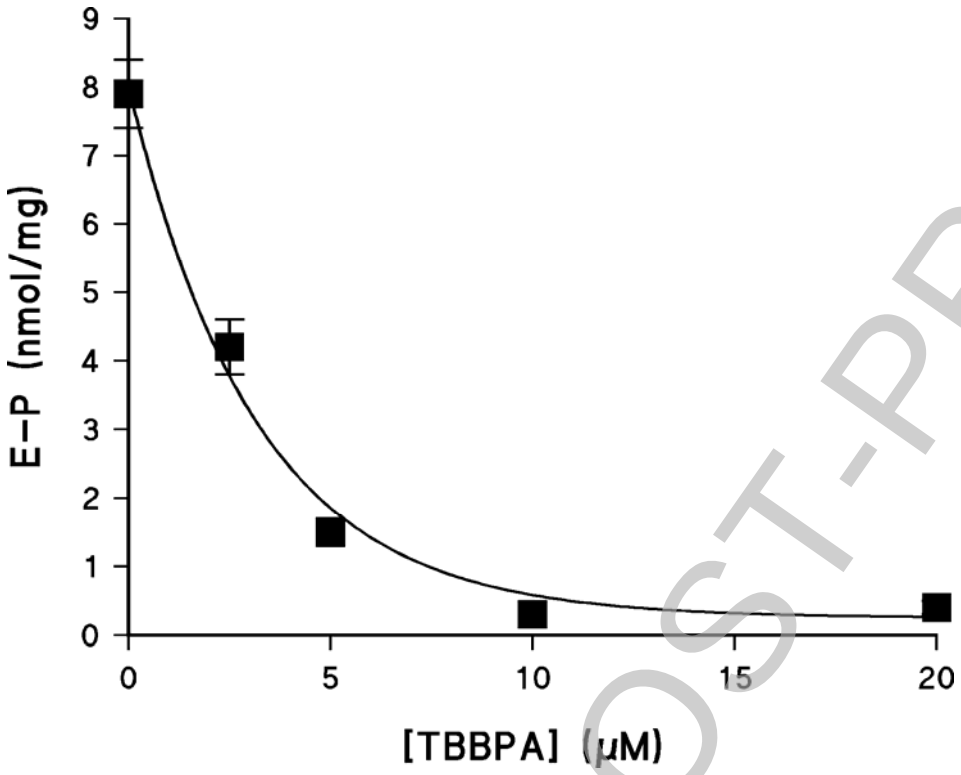
FIG 3

A



B

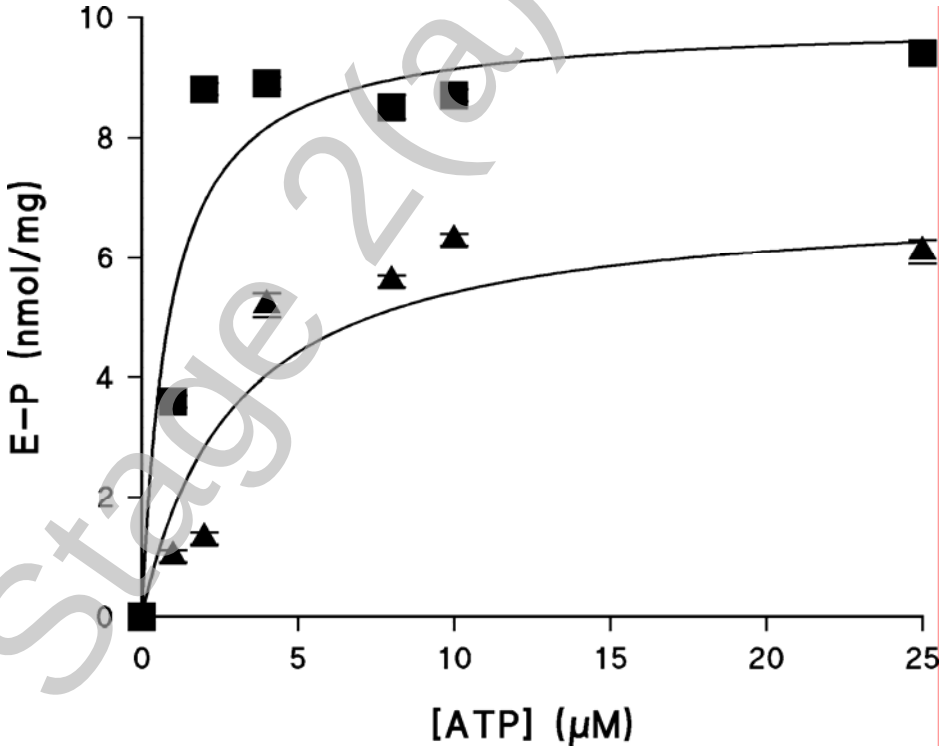


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FIG 4

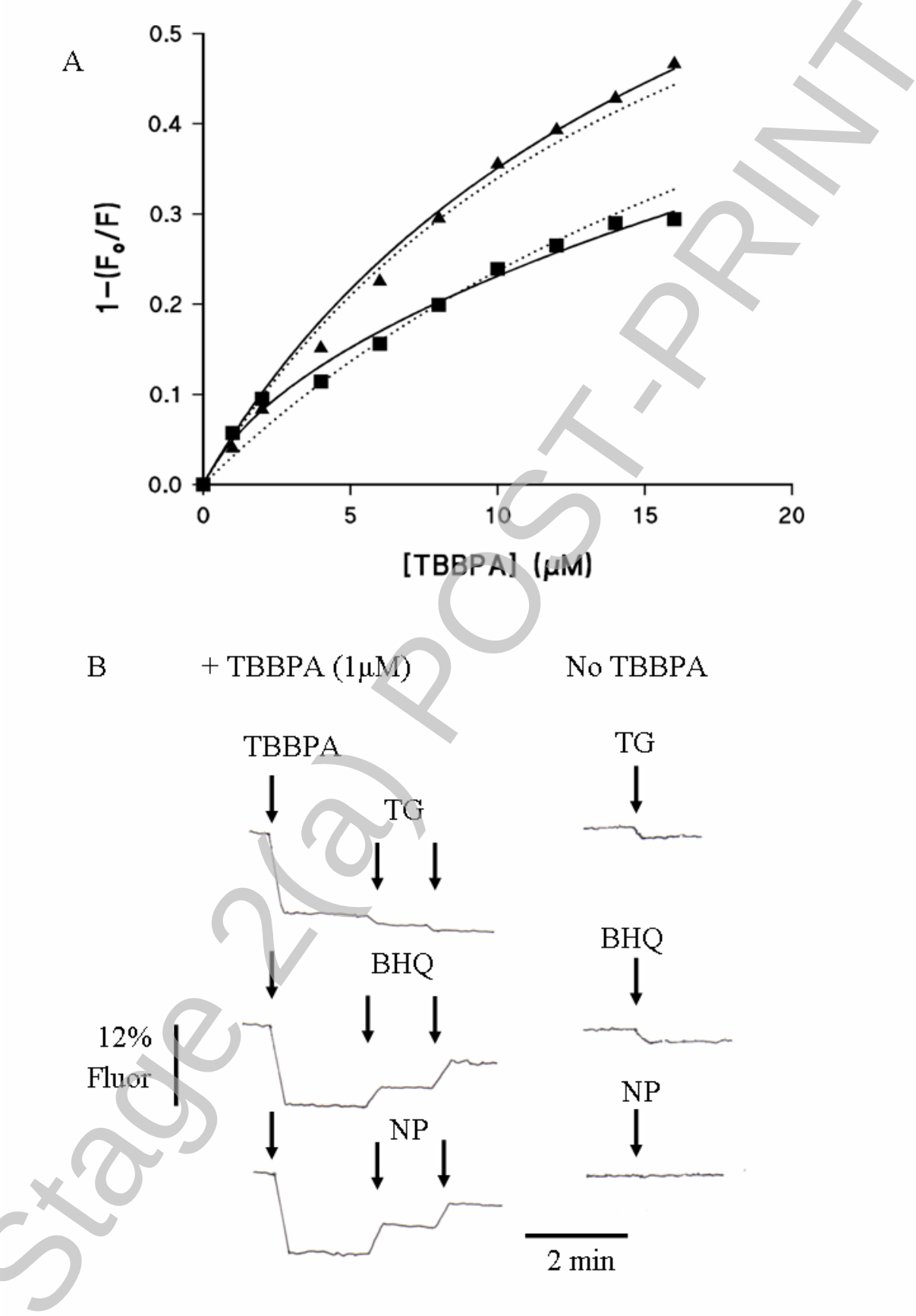

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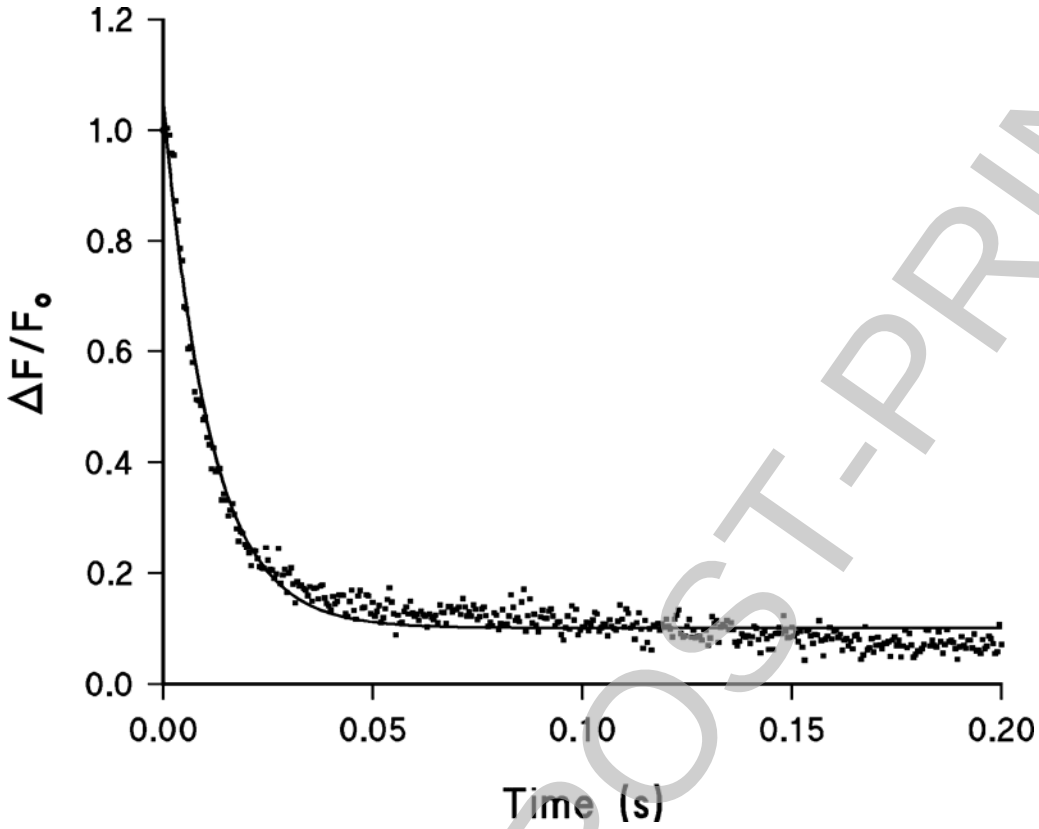
FIG 5

A



B

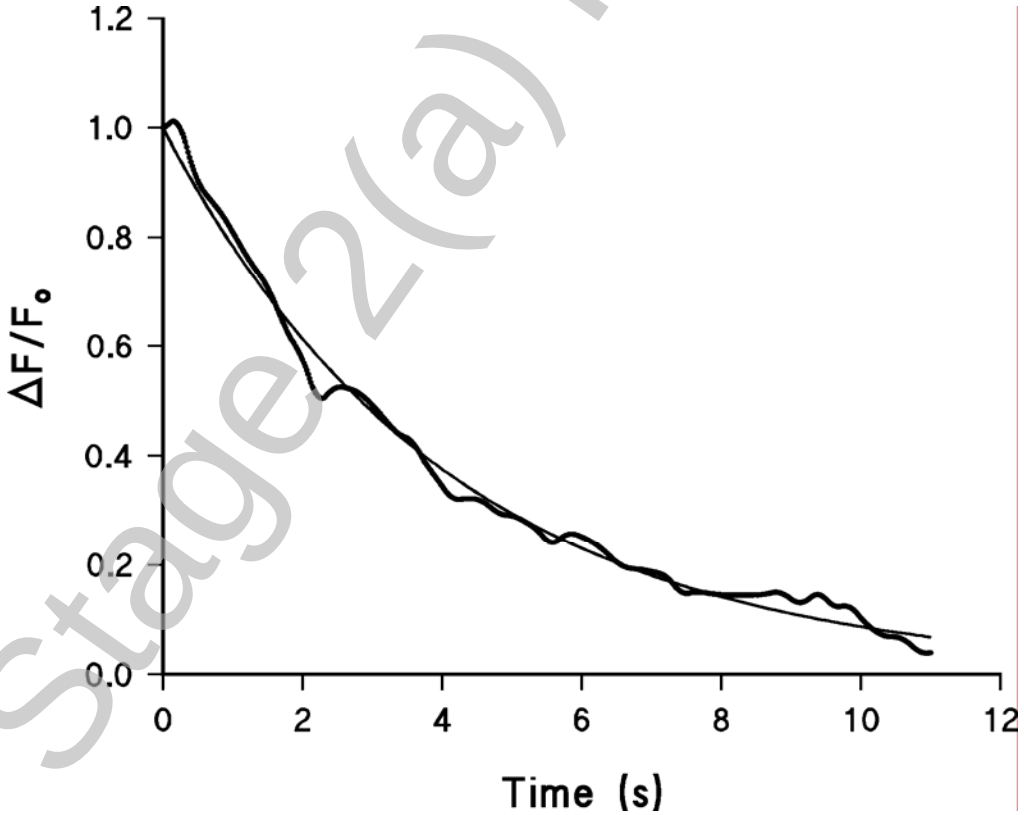


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FIG 6

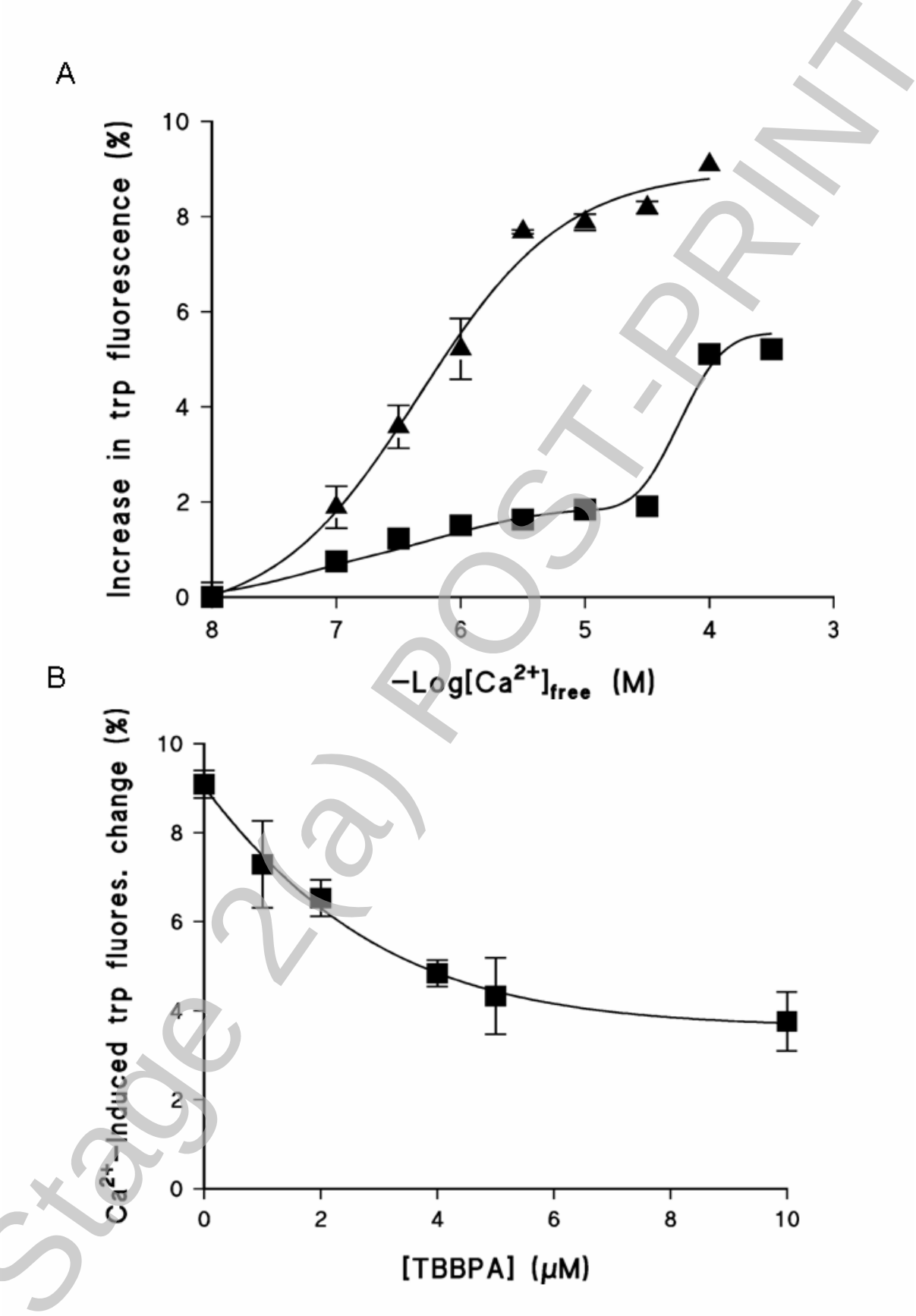

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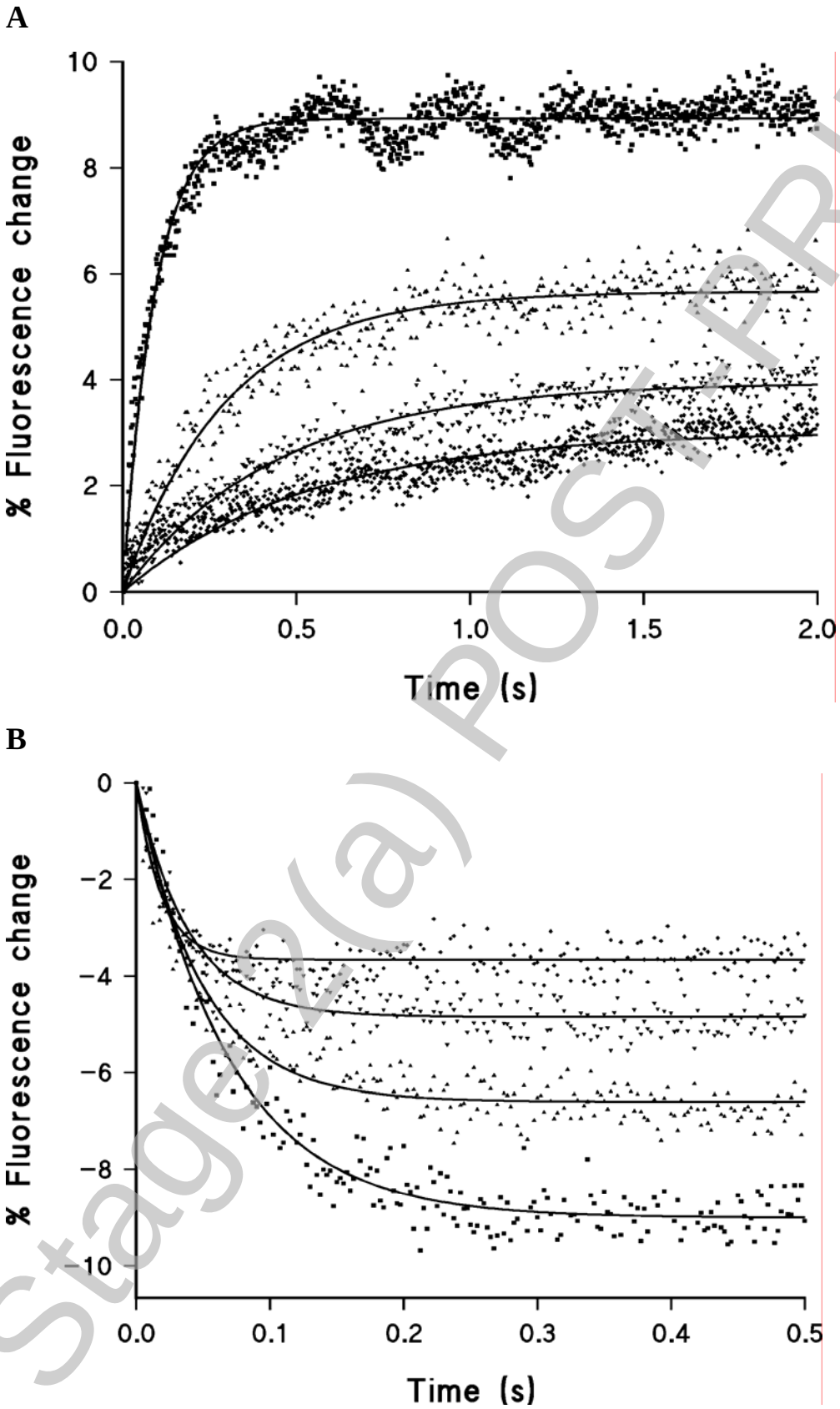
FIG 7



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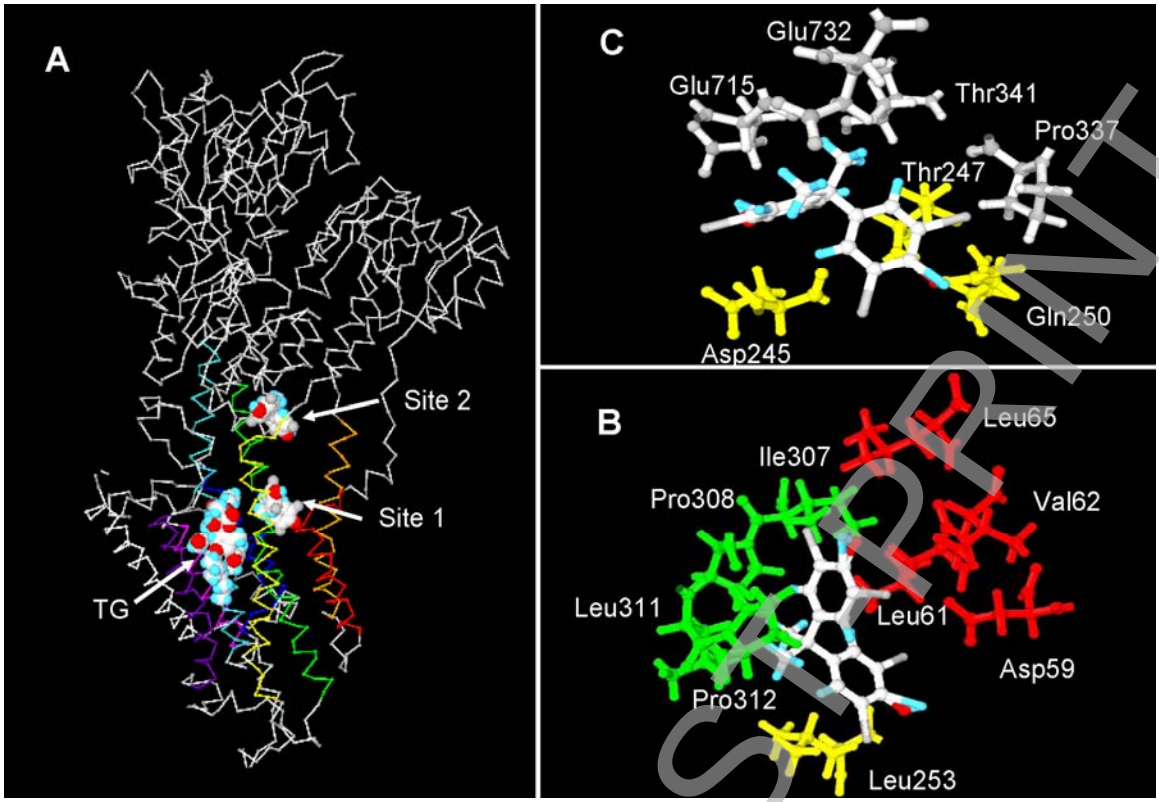

FIG 8

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