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The Interaction of an impermeant cation with the sheep Cardiac RyR Channel Alters Ryanoid Association.

Bhavna Tanna, William Welch, Luc Ruest, John L. Sutko & Alan J Williams

Cardiac Medicine, National Heart & Lung Institute, Imperial College London, Guy Scadding Building, Dovehouse Street, London SW3 6LY, UK. (B.T. & A.J.W.)

Department of Biochemistry (W.W.) and Department of Pharmacology (J.L.S.), University of Nevada School of Medicine, Reno, Nevada 89557, U.S.A.

Department of Chemistry, University of Sherbrooke, Quebec J1K 2R1, Canada. (L. R.)

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Running Title: Effects of a blocking cation on ryanoid interaction with RyR

Correspondence to:

Professor Alan J. Williams

Cardiac Medicine, National Heart & Lung Institute, Imperial College London,

Guy Scadding Building, Dovehouse Street,

London, SW3 6LY. UK

Phone: +44 (0)207 351 8137

Fax: +44 (0)207 823 3392

Email: a.j.williams@imperial.ac.uk

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Abstract

In earlier studies we have demonstrated that the interaction of ryanoids with the sarcoplasmic reticulum Ca^{2+}-release channel (Ryanodine Receptor (RyR)) incorporated into planar lipid bilayers reduced the effectiveness of the tetraethylammonium (TEA^+) as a blocker of K^+ translocation (Tanna et al., 2001). In this communication we have investigated both the effect of TEA^+ on [^{3}H]-ryanodine binding and the actions of this impermeant cation on the interaction of the reversible ryanoid, 21-amino-9α-hydroxyryanodine with individual, voltage clamped, RyR channels. A dose-dependent inhibition of [^{3}H]-ryanodine binding was observed in the presence of TEA^+, suggesting that the cation and alkaloid compete for access to a common site of interaction. Single channel studies gave further insights into the mechanism of the competition between the two classes of ligands. TEA^+ decreases the association rate of 21-amino-9α-hydroxyryanodine with its receptor, whilst the dissociation rate of the ryanoid from the channel was unaffected. Our results demonstrate that TEA^+ inhibits both K^+ translocation through RyR, and ryanoid interaction at the high affinity ryanodine site on the channel. These actions involve binding of TEA^+ to different, but weakly interacting, sites in the RyR channel.

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Introduction

The plant alkaloid ryanodine binds with high affinity to a class of large homotetrameric (monomer ~550kDa) intracellular membrane Ca^{2+}-release channels, referred to as the ryanodine receptor (RyR) and alters channel function dramatically (Sutko and Airey, 1996;Sutko et al., 1997). Channel open probability (P_o) increases and the rate at which ions transverse the channel is significantly reduced (Rousseau et al., 1987;Lindsay et al., 1994;Tinker et al., 1996). The characteristically reduced conductance states observed upon the interaction of ryanodine and its derivatives (ryanoids) (Tinker et al., 1996) with the RyR reflect alterations in both the relative permeability of ions and affinity of sites within the channel for these ions (Lindsay et al., 1994). The interaction of a ryanoid with RyR induces a conformational change in the pathway through which ions are translocated (the channel pore) by stabilizing a conformation of the pore that is not normally observed in the absence of a ryanoid (Tanna et al., 2001;Tanna et al., 2005). The location of the high affinity ryanodine binding site is still the subject of much speculation; it has not yet been established whether the structural reorganisation of the pore region of the protein takes place upon the interaction of ryanoids directly within this structure or elsewhere on the protein resulting in an allosteric effect.

However, a considerable amount of information appears to be consistent with the proposal that the ryanoid interaction site is located within the pore of the RyR channel. Studies involving proteolytic degradation and photo-affinity labelling have demonstrated that the ryanodine binding site is localised to a 76-kD region of the skeletal RyR at the carboxyl terminus (Callaway et al., 1994;Witcher et al., 1994).

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Trans-membrane helices including the pore forming components of the channel molecule are located in this region (Balshaw et al., 1999;Williams et al., 2001;Welch et al., 2004). Point mutation of various residues within the pore forming region of the RyR produced alterations in the interaction of both [^{3}H]-ryanodine with populations of receptors and ryanoids with single channels, without altering other characteristics of channel function (Chen et al., 2002;Wang et al., 2003;Ranatunga et al., 2005). Consistent with a location of the high affinity ryanoid binding site within the pore of the RyR channel, [^{3}H]-ryanodine binding studies and single channel experiments have established that ryanodine binds preferentially to the open conformation (Chu et al., 1990;Meissner and El-Hashem, 1992;Tanna et al., 1998) and that the site is only available from the cytosolic face of the channel (Tanna et al., 1998). More specifically, interaction of ryanoids with the RyR is voltage dependent, the rate of ryanoid association (k_{on}) with the channel increases, whilst the rate of ryanoid dissociation (k_{off}) from the channel decreases as trans-membrane holding potential is taken to high positive values (Tanna et al., 1998;Tanna et al., 2000;Tanna et al., 2003). This influence of trans-membrane holding potential on ryanoid interaction with the channel is largely due to a voltage dependent alteration in the affinity of the channel for ryanoids; however the net charge of the ryanoid also contributes to the overall voltage dependence of the interaction of a ryanoid with the RyR and is in the order cationic > neutral > anionic indicating that the site of interaction may be within the voltage drop across the channel pore (Tanna et al., 2000;Tanna et al., 2003).

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If the high affinity ryanoid binding site is located within the voltage drop across the pore of the channel, it is possible that an impermeant cation that interacts at a site within the voltage drop may impede the passage of a ryanoid to its binding site. Inhibition of charybdotoxin interaction with Ca^{2+}-activated K^+ channels by TEA^+ provides a precedent for such a mechanism (Miller, 1988). In this communication we have monitored the influence of an impermeant cation, that interacts within the voltage drop across RyR, on [^{3}H]-ryanodine binding. In addition we have assessed the mechanism by which an impermeant ion alters the interaction of ryanoids with RyR by monitoring interactions of a rapidly dissociating ryanoid with single channels in the absence and presence of TEA^+.

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Materials and Methods

Materials

Phosphatidylethanolamine was supplied by Avanti Polar Lipids, Inc. and phosphatidylcholine by Sigma-Aldrich. [^{3}H]-ryanodine was purchased from New England Nuclear Ltd. Aqueous counting scintillant was purchased from Packard. Standard chemicals were obtained as the best available grade from BDH Ltd. or Sigma-Aldrich. 21-amino-9α-hydroxyryanodine was synthesized as described earlier (Welch et al., 1997) and stored as a stock solution in 50% aqueous ethanol at -20°C.

Isolation of Sheep Cardiac Heavy Sarcoplasmic Reticulum Membrane Vesicles

Heavy sarcoplasmic reticulum (HSR) membrane vesicles were prepared using procedures described earlier (Sitsapesan and Williams, 1990). A mixed membrane fraction was obtained by differential centrifugation after homogenisation of the ventricular septum and left ventricular free wall. The mixed membrane vesicles were further fractionated by sucrose density gradient centrifugation and the HSR fraction collected at the 30 / 40% (wt/vol) interface. The HSR fraction was resuspended in 0.4 M KCl before sedimentation at 100,000 *g*. The resulting pellet was resuspended in 0.4 M sucrose, 5 mM HEPES, titrated to pH 7.2 with tris hydroxymethyl methylamine (Tris) and frozen and stored in liquid N_2. The method of Bradford (Bradford, 1976) was used to assay the concentration of protein in all membrane samples.

Solubilisation and Separation of the Ryanodine Receptor

HSR membrane vesicles were solubilised with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) and RyR was isolated and

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reconstituted into unilamellar liposomes for incorporation into planar phospholipids bilayers, as described previously (Lindsay and Williams, 1991).

[^{3}H]-Ryanodine Binding Assay

HSR membrane vesicles were diluted to 50-100 μg protein / ml of which 10 μl were incubated with 5 nM [^{3}H]-ryanodine at 37°C with constant shaking in a buffer medium, designed to optimise channel open probability, containing 1 M KCl, 1 mM ATP, 100 μM total Ca^{2+}, 10 mM PIPES (pH 7.4). The samples were incubated for the times indicated in the text ± TEA^+ at the concentrations also indicated in the text (stock solutions of which, were made up in buffer medium), in a final volume of 1 ml. All assays were performed in triplicate and the number of separate HSR preparations is detailed in the results section. Non-specific binding was determined from duplicate assays in the presence of a 1000 fold excess (5 μM) of unlabelled ryanodine. Binding was terminated by the addition of 5 ml of ice-cold buffer medium followed immediately by filtration through Whatman GF-B filters pre-soaked in buffer medium. To remove residual unbound [^{3}H]-ryanodine the filters were washed with a further two aliquots of buffer medium. Radioactivity remaining on the filter was determined by placing the filter in 10 ml aqueous UltraGold MV scintillant (Packard). Vials were vortexed and left to soak for at least 24 hours to allow the filters to equilibrate with the scintillant before [^{3}H]-ryanodine was quantified by liquid scintillation counting. Specific [^{3}H]-ryanodine binding data are displayed as pmoles of [^{3}H]-ryanodine bound per mg of membrane protein.

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Data were analysed and displayed using PRISM (Graph Pad Prism, San Diego, CA USA). The binding obtained with increasing blocker concentration was fitted with equation *1*, describing a sigmoidal inhibition of binding activity.

$$b = \frac{b_0}{1 + ([blocker]) / IC_{50})^n} \qquad (1)$$

Where *b* is the [^{3}H]-ryanodine bound at [blocker] and b_o represents maximum binding i.e. in the absence of added blocker, IC_{50} represents the concentration of blocker at which b is 50% of the maximum. *n* is the Hill coefficient.

Planar Phospholipid Bilayers

Phospholipid bilayers were formed from suspensions of phosphatidylethanolamine in n-decane (35 mg/ ml) across a 200-μm diameter hole in a polystyrene copolymer partition that separated two chambers referred to as *cis* (0.5 ml) and *trans* (1.0 ml). The *trans* chamber was held at virtual ground while the *cis* chamber could be clamped at holding potentials relative to ground. Current flow across the bilayer was monitored using an operational amplifier as current-voltage converter (Miller, 1982). Bilayers were formed with solutions containing 600 mM KCl, 20 mM HEPES, titrated to pH 7.4 with KOH, resulting in a solution containing 610 mM K^+ in both chambers. An osmotic gradient was created by the addition of an aliquot (50-100 μl) of 3 M KCl to the *cis* chamber. Proteoliposomes were added to the *cis* chamber and stirred. Under these conditions, channels usually incorporated into the bilayer within 2-3 min. If channels did not incorporate, a second aliquot of 3 M KCl could be added to the *cis* chamber. After channel incorporation, further fusion was prevented by perfusion of

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the *cis* chamber with 610 mM K^+. Channel proteins incorporate into the bilayer in a fixed orientation so that the cytosolic face of the chamber is exposed to the solution in the *cis* chamber and the luminal face of the channel to the solution in the *trans* chamber. Single channel P_o was increased by the addition of up to 100 μM EMD 41000 to the cytosolic face of the channel (McGarry and Williams, 1994;Tanna et al., 1998). Only bilayers containing a single channel were used in the experiments described in this communication. Experiments were carried out at room temperature (21 ± 2°C).

The interaction of 21-amino-9α-hydroxyryanodine with the channel was studied by adding the indicated concentration to the solution at the cytosolic face of the bilayer. Although TEA^+ is an effective blocker of the RyR channel only from the cytosolic face of the channel (Lindsay and Williams, 1991), it was added symmetrically to the solutions at both sides of the bilayer to avoid the possibility of asymmetric surface potential arising from the binding of the cation to the bilayer.

Single Channel Data Acquisition

Single channel current fluctuations were displayed on an oscilloscope and stored on Digital Audio Tape. For analysis, data were replayed, filtered at 1 kHz with an eight-pole Bessel filter, and digitised at 4 kHz using Satori V3.2 (Intracel, Cambridge, UK). Single channel current amplitudes and lifetimes were measured from digitized data. The representative traces shown in the figure were obtained from digitised data acquired with Satori V3.2 and transferred as HPGL graphics software package (CorelDraw; Corel Systems Corp., Ottawa, Canada) for annotation and printing.

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The Analysis of the Interaction of 21-amino-9α-hydroxyryanodine with Single RyR Channels

21-amino-9α-hydroxyryanodine interacts reversibly with the high affinity ryanodine-binding site on the SR Ca^{2+}-release channel and induces modifications of channel function; single channel conductance is reduced in the presence of the permeant cation K^+ and channel P_o is increased (Tanna et al., 1998). Previously we have established that the interaction of 21-amino-9α-hydroxyryanodine with the channel and the resulting modification of channel function can be described by a simple bimolecular reaction scheme (Tanna et al., 1998). As a consequence, apparent rate constants for the association (k_{on}) and dissociation (k_{off}) of 21-amino-9α-hydroxyryanodine can be determined from the mean dwell times in the unmodified and modified conductance states (Equation *2* and *3*):

$$k_{on} = (\tau_{unmod})^{-1} \quad (2)$$

and

$$k_{off} = (\tau_{mod})^{-1} \quad (3)$$

Dwell times and the probability that the channel is in the ryanoid-modified state (P_{mod}) were determined by using Satori V3.2 as described previously (Tanna et al., 1998). Sections of the data were defined as the unmodified state (periods where the channel displayed transitions between the open and the closed levels) or the modified state (periods in which the channel displayed transitions between the modified and the closed levels). To obtain sufficient events, these parameters were obtained from steady state recordings lasting at least 6 minutes.

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The Probability of the Channel Being in the Open State

While the rate of 21-amino-9α-hydroxyryanodine dissociation from the RyR is independent of P_o, the rate of association of the ryanoid with the channel is directly proportional to channel P_o (Tanna et al., 1998). For this reason, it was necessary to measure P_o in all experiments. This was done by monitoring this parameter in the sections of the recorded data during which no ryanoid was bound; i.e., with transitions only between the open and closed conductance levels. P_o was determined by 50% threshold analysis as described previously (Sitsapesan and Williams, 1994). To minimize variability in P_o, all experiments were carried out in the presence of cytosolic EMD 41000. The k_{on} values quoted for 21-amino-9α-hydroxyryanodine have been normalised to a P_o of 1.0 (Tanna et al., 1998).

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Results

As suggested in the introduction, a number of observations arising from previous investigations are consistent with the proposal that the high affinity ryanodine binding site is located within the pore of RyR, and possibly some way into the voltage drop across the channel. In this communication we have investigated this hypothesis by a) examining the interaction of [^{3}H]-ryanodine with populations of receptors in the presence of TEA^+, an impermeant cation that interacts at a site within the voltage drop across the channel, and b) monitoring the influence of this blocking cation on the interaction of the reversible ryanoid, 21-amino-9α-hydroxyryanodine with individual channels under voltage clamp conditions.

Does TEA^+ Influence the interaction of [^{3}H]-Ryanodine with the High Affinity Ryanodine Binding Site?

TEA^+ is a concentration- and voltage-dependent blocker of K^+ conductance in the RyR channel and is effective only from the cytosolic side of the channel (Lindsay et al., 1991;Tinker et al., 1992). TEA^+ binds to a site approximately 90% into the voltage drop across the channel from the cytosolic face of the membrane and gives rise to unresolved blocking events which produce a time averaged reduction in single channel conductance (Lindsay et al., 1991). If the high affinity ryanodine/ryanoid binding site is located within the voltage drop across the channel, it is possible that impermeant cations such as TEA^+, which interact within this region, would, by some mechanism, impede the passage of a ryanoid to this site. To investigate this hypothesis we monitored [^{3}H]-ryanodine binding in the absence and presence of the impermeant cation.

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The effect of the blocking cation on ryanodine binding is shown in Figure 1. The curves on this plot are best fits to exponential association using the relationship:

$$\text{Ryanoid bound} = B_i\,(1 - \exp(-t/\tau)) \qquad (4)$$

where B_i is the amount of ryanoid bound at infinite time and τ is the apparent relaxation time ($1/\tau = k_{on}$ [ryanodine] + k_{off}) obtained at fixed concentrations of ryanodine and TEA^+. In the absence of TEA^+, the amount of ryanodine bound at equilibrium (B_i) is 0.51 ± 0.03 pmol/mg protein with a relaxation time of 9 ± 1 minutes. The presence of 500 mM TEA^+ reduces the amount of ryanodine bound at equilibrium to 0.39 ± 0.03 pmol/mg protein and increases the relaxation time to 22 ± 5 minutes.

To characterise the influence of the blocking cation, samples were incubated with increasing concentrations of blocker for 10 minutes. Under these conditions TEA^+ reduces [^{3}H]-ryanodine binding in a concentration-dependent manner as demonstrated in Figure 2. The solid line in Figure 2 is a best fit to Equation *1* obtained by non-linear regression, giving an IC_{50} value and a Hill coefficient of 668 mM and 1.42 respectively. These data indicate that the presence of an impermeant cation reduces the probability of interaction of [^{3}H]-ryanodine with the RyR channel. To obtain an insight into the mechanism that governs this process, single channel experiments were carried out.

Single channel studies have demonstrated that the interaction of the cationic ryanoid, 21-amino-9α-hydroxyryanodine (formal charge +1) with RyR is dependent upon ligand concentration and trans-membrane holding potential. However, unlike

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ryanodine, on the time scale of a single channel experiment, 21-amino-9α-hydroxyryanodine interacts with the RyR channel reversibly, leading to periods of normal channel gating to only the open and closed level, and durations when the channel is in a modified level from which it may close with the ryanoid bound (Tanna et al., 1998). The properties of 21-amino-9α-hydroxyryanodine made it a useful ligand with which to assess if the presence of TEA^+ influenced the interaction of a ryanoid with the RyR channel.

The Influence of TEA^+ on the Interaction of 21-Amino-9α-Hydroxyryanodine with Single RyR Channels

The interaction of 21-amino-9α-hydroxyryanodine with the RyR channel was monitored in the absence and presence of TEA^+. Figure 3 shows the influence of increasing TEA^+ concentrations on the probability of the interaction of 500 nM 21-amino-9α-hydroxyryanodine with a single RyR channel at a holding potential of 40 mV. In the absence of added TEA^+ the characteristic reversible modification of RyR channel gating and ion handling by 21-amino-9α-hydroxyryanodine is observed; the interaction of the ryanoid with the channel results in the occurrence of clearly defined events in which unitary conductance is reduced and the probability of channel closing is reduced dramatically (Tanna et al., 1998). The presence of increasing concentrations of TEA^+ modifies various aspects of RyR channel function. As expected, TEA^+ produces a concentration-dependent reduction of K^+ conductance in the RyR channel when 21-amino-9α-hydroxyryanodine is not bound (Lindsay et al., 1991;Tinker et al., 1992). TEA^+ produces a qualitatively similar reduction in K^+ conductance in the ryanoid modified state of the channel; however the degree of block

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is noticeably lower than that seen in the unmodified channel. Clearly, while 21-amino-9α-hydroxyryanodine interacts with its binding site on the RyR channel and alters the manner in which ions are translocated through the channel, TEA^+ is able to enter the voltage drop and reduce K^+ conductance with the ryanoid bound (Tanna et al., 1998;Tanna et al., 2001).

In addition to modifying ion translocation through the RyR channel, TEA^+ also influences the likelihood of the interaction of 21-amino-9α-hydroxyryanodine with its binding site on the channel. The probability of channel modification by 21-amino-9α-hydroxyryanodine is markedly reduced by the increasing concentrations of TEA^+ (Figure 3). The influence of TEA^+ concentration on the P_{mod} of a number of individual channels at a holding potential of +40 mV is shown in Figure 4. P_{mod} declines from 0.70 ± 0.03 at 0 mM TEA^+ to 0.41 ± 0.06 in the presence of 80 mM TEA^+. An inspection of the apparent rate constants for the association and dissociation of 21-amino-9α-hydroxyryanodine with increasing concentrations of TEA^+ (Figure 5), indicates that the reduction in P_{mod} illustrated in Figure 4 results from a concentration-dependent linear reduction in k_{on} with a slope of $-18.59 \pm 2.19\ s^{-1}\ mM^{-2}$. Alterations in TEA^+ concentrations had no significant effect on k_{off} (the mean value across the concentration range is $0.23 \pm 0.02\ s^{-1}$). The data indicate that reduced binding of [^{3}H]-ryanodine to populations of RyR channels (Figure 1 & 2) is likely to result from a lowering in the rate of ryanoid association in the presence of TEA^+.

Mechanisms underlying the inhibition of K^+ translocation and [^{3}H]-ryanodine binding by TEA^+

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The data presented in this communication demonstrate that in addition to its well characterised action as a blocker of K^+ translocation in the RyR2 channel, TEA^+ also inhibits the interaction of [^{3}H]-ryanodine to the high affinity binding site on this channel. In comparing the mechanisms involved in these two actions of TEA^+ we have initially determined the number of ions involved in the respective reactions.

The interaction of TEA^+ with RyR produces a non-conducting or blocked form of the channel (RyRNC). In the simplest case, block will result from the occupancy of the channel pore by a single TEA^+. In this case the reaction can be described as:

$$RyR + TEA^+ = RyRNC \qquad (5)$$

Alternatively block may require the interaction of more than one TEA^+; in which case the reaction will be described by:

$$RyR + nTEA^+ = RyRNC \qquad (6)$$

Ignoring any intermediate, conducting forms, the resulting equilibrium constants are:

$$K_{eq} = [RyRNC]/([RyR]\,[TEA^+]) \qquad (7)$$

or

$$K_{eq} = [RyRNC]/([RyR]\,[TEA^+]^n) \qquad (8)$$

To describe [^{3}H]-ryanodine (Ry) binding to RyR in the presence of TEA^+ one may write

$$RyR + Ry = RyR\bullet Ry \qquad (9)$$

$$RyR + TEA^+ = RyR\bullet TEA^+ \qquad (10)$$

$$RyR\bullet TEA^+ + TEA^+ = RyR\bullet TEA^+_2 \qquad (11)$$

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If RyR●TEA^+ cannot bind ryanodine, then the inhibition of [^{3}H]-ryanodine binding will be first order with respect to TEA^+ concentration. However, if RyR●TEA^+ can bind [^{3}H]-ryanodine but RyR●TEA^+_2 cannot, then the following reaction is possible.

$$RyR\bullet TEA^+ + Ry = RyR\bullet TEA^+\bullet Ry \qquad (12)$$

In such a case, TEA^+ inhibition of ryanodine binding will be second order in TEA^+ concentration. Ignoring any intermediate ryanodine binding forms, the corresponding equilibrium constant is

$$K_{eq} = [RyR\bullet TEA^+_2]/([RyR]\,[TEA^+]^2) \qquad (13)$$

By analysis of the data presented in this communication we can provide some evidence towards determining if, in the presence of TEA^+, the non-conducting form of RyR is the same as the form of RyR that cannot bind [^{3}H]-ryanodine.

The data in Figure 3 demonstrates block of K^+ translocation through RyR by TEA^+ from the cytosolic face of the channel. Mean data for experiments such as that shown in Figure 3 were expressed as the fraction of RyR in the non-conducting form; f, where

$$f = (C_o - C)/(C_o - C_i) = [RyRNC] / ([RyR] + [RyRNC]) \qquad (14)$$

C_o is the conductance in the absence of TEA^+, C_i is the conductance at a saturating concentration of TEA^+ and C is the measured conductance with varying TEA^+ concentration. Fractional change is related to the apparent equilibrium constant by combining equations *8* and *14*

$$f / (1-f) = [TEA^+]^n K_{eq} \qquad (15)$$

or in logarithmic form

$$\log (f/(1-f)) = n \log [TEA^+] - \log (K_{eq}) \qquad (16)$$

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where n is the order of the reaction. A plot of log [f/(1-f) versus log [TEA^+] will have a Y-intercept equal to –log (K_{eq}) and a slope equal to n.

In Figure 6A, the effect of TEA^+ concentration on the open channel conductance is presented as a log-log plot. The slope of the line is 1.0 ± 0.1 indicating a first order relationship between TEA^+ concentration and block of K^+ current. A similar treatment of the effect of TEA^+ concentration on the conductance of the channel modified by the interaction of 21-amino-9α-hydroxyryanodine (see Figure 3) is shown in Figure 6B and produces a slope of 1.2 ± 0.1 again indicating a first order relationship between TEA^+ concentration and block of K^+ current. By avoiding the long extrapolation, the dissociation constants ($K_D = 1/K_{eq}$) are better determined by direct plots of K^+ conductance versus TEA^+ concentration (not shown). The dissociation constants obtained in this manner are 0.031 ± 0.002 M and 0.061 ± 0.007 M for the unmodified and ryanoid-modified RyR respectively.

The dose-response relationship between TEA^+ concentration and [^{3}H]-ryanodine binding is shown in Figure 2. Analysis of this data as described above (using the relationship log (f/(1-f)) = n log [TEA^+] – log (K_{eq})) produces a plot with a slope of 2.14 ± 0.24 indicating that, in contrast to the inhibition of K^+ translocation, the inhibition of the binding of [^{3}H]-ryanodine requires the interaction of two TEA^+s (Figure 7).

The ability of TEA^+ to alter the conductance of the ryanoid-modified state of the RyR indicates that both TEA^+ and ryanoid can occupy the channel simultaneously. Yet,

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the ryanoid binding studies indicate the TEA^+ and ryanoid are mutually exclusive. This apparent contradiction can be resolved by proposing two binding sites for TEA^+: one site is also a K^+ binding site required for cation translocation; the other site is within the ryanoid binding site. The first order relationship between TEA^+ concentration and K^+ conductance indicates that binding of a single TEA^+ is sufficient to block K^+ conductance. In contrast the binding of two TEA^+s are required to block ryanoid binding (see below). The difference in order is a further indication of two separate sites for TEA^+ binding. If binding of TEA^+ to the K^+ site were independent of ryanoid binding, the apparent dissociation constant of TEA^+ would be independent of the presence of ryanoid. However, occupation of the ryanoid-binding site increases the dissociation constant of TEA^+ two-fold (a difference 4.3 times the standard error of the mean). Using the Gibbs free energy relationship ($\Delta G^o = -RT \ln(K_{eq})$) one finds a difference in free energy of 0.4 kcal/mol, a weak interaction comparable to a hydrogen bond.

The single channel data indicate that TEA^+ and ryanoid can bind to the channel simultaneously; therefore binding of the two ligands is not mutually exclusive. Figures 1 and 2 show that TEA^+ inhibits the binding of ryanoid and extrapolation of the data indicates that at infinite TEA^+ ryanoid does not bind. The weak interaction between TEA^+ bound at the blocking site and the ryanoid binding site (see above) would suggest that complete abolition of ryanoid binding could not be a consequence of saturation of the TEA^+ cation-translocation blocking site, rather it is consistent with the existence of at least two, non-identical, TEA^+ binding sites. The inference is

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consistent with Figure 3 where TEA^+ alters the conductance of both the open and the ryanoid-modified RyR channel.

A potential mechanism for the inhibition of both permeant ion translocation and ryanoid binding by TEA^+ is outlined in Scheme 1.

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Discussion

Various lines of evidence (described in the Introduction to this work) are consistent with the proposal that the high affinity ryanoid binding site is located within the conduction pathway or pore of the RyR channel. Here we have investigated the hypothesis that TEA^+, an impermeant cation that blocks the translocation of permeant cations, could influence the interaction of ryanodine and its derivatives with this putative binding site by occupying its well characterised blocking site located towards the luminal extremity of the voltage drop across the RyR channel pore. Our experiments demonstrate that, in addition to blocking K^+ translocation, TEA^+ inhibits both the interaction of [^{3}H]-ryanodine with isolated HSR membrane vesicles and the association of 21-amino-9α-hydroxyryanodine with individual RyR channels under voltage clamp conditions.

We have identified three potential mechanisms that might underlie the actions of TEA^+ reported here. It has been well documented that [^{3}H]-ryanodine binding to populations of RyR channels is altered by interventions that modify RyR open probability; ligands that activate the channel such as Ca^{2+}, caffeine and ATP increase equilibrium binding whilst ligands that lower channel open probability, such as Mg^{2+} and ruthenium red, reduce binding (Coronado et al., 1994;Meissner, 1994;Zucchi and Ronca-Testoni, 1997;Shoshan-Barmatz and Ashley, 1998). Therefore one potential mechanism is that the observed reduction in binding of [^{3}H]-ryanodine reflects a lowering of channel P_o by TEA^+.

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This possibility was investigated by monitoring the interactions of a rapidly dissociating ryanoid, 21-amino-9α-hydroxyryanodine, with individual RyR channels in the absence and presence of TEA^+ under conditions where channel P_o was monitored continuously. Any potential influence of channel Po was eliminated in these experiments by determining rates of ryanoid association and dissociation following normalisation of any minor variations in P_o (Tanna et al., 1998). Consistent with the observed reduction in [^{3}H]-ryanodine binding, single channel experiments demonstrate that increasing concentrations of TEA^+ reduce the likelihood of channel modification by 21-amino-9α-hydroxyryanodine. Further, this altered probability of interaction results from a reduced rate of ryanoid association with increasing concentration of TEA^+ while the rate of dissociation of 21-amino-9α-hydroxyryanodine from RyR is unaffected by TEA^+. As a consequence we can conclude that the reduced probability of ryanoid interaction with RyR is mediated by a direct action of TEA^+, independent of variations in channel P_o.

Our remaining hypothetical mechanisms for the observed inhibition of ryanoid interaction by TEA^+ are that either TEA^+ bound to the K^+ translocation blocking site inhibits ryanoid interaction with RyR or that inhibition of ryanoid binding by TEA^+ occurs as the result of TEA^+ interaction with a site other than the K^+ translocation blocking site. The kinetic analysis of block and ryanoid binding presented in the results section of the communication excludes the former and is consistent with the latter mechanism and, as a consequence, we propose that the inhibition by TEA^+ of K^+ conductance and ryanoid binding requires at least two TEA^+ binding sites. One site binds K^+ as an essential part of ion translocation, whereas the other site is part of the

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high affinity ryanoid binding site. The binding of a single TEA^+ to the K^+ site is sufficient to block conductance; in contrast, block of ryanodine binding requires the interaction of two TEA^+s. The two TEA^+ binding sites (K^+ blocking and ryanoid interaction) are in weak communication with an interaction energy of ~0.4 kcal/mol. The perturbation of TEA^+ binding by ryanoid is equivalent to the perturbation of ryanoid binding by TEA^+, consistent with the thermodynamics of interacting sites.

Our experiments have revealed a novel interaction between TEA^+ and the high affinity ryanoid binding site in RyR and have established that this site is distinct from the site at which TEA^+ blocks K^+ translocation in this channel. What do these observations add to our attempts to test the hypothesis that the high affinity ryanoid binding site is located in the pore of RyR? The pore region of the RyR channel, as proposed in a recent model in which the KcsA K^+ channel was used as a template, consists of a wide cytosolic cavity and a more constricted region, equivalent to the selectivity filter of a K^+ channel, which connects the cytosolic cavity with the lumen of the sarcoplasmic reticulum (Welch et al., 2004). By analogy with K^+ channels we presume that the bulk of the voltage drop across the RyR will occur in this constricted region. The K^+-translocation blocking site of TEA^+ is within the pore of the RyR channel. More specifically, blocking experiments indicate that this site is located deep within the voltage drop across the pore (Lindsay et al., 1991) and, consistent with this, molecular dynamics simulations within the model of the RyR pore suggest that this site is positioned towards the luminal extremity of the constricted region of the pore formed by residues equivalent to the signature selectivity sequence of K^+ channels (Welch et

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al., 2004). Data presented here indicate that there is a degree of communication between this conduction-blocking site and the high affinity ryanoid site.

An increasing number of lines of evidence are consistent with a proposed location for the high affinity ryanoid binding site within the pore of the RyR channel (see above). The novel data reported here, whilst not providing unequivocal evidence for the location of this site, either within the pore or elsewhere on RyR, do allow us to refine this proposition by concluding that the ryanoid binding site is not positioned deep within the narrowest region of the pore. If the ryanoid binding site is within the pore, a more probable site of interaction may be provided by the inner helices that line the cytosolic cavity of the pore in the open channel (Welch et al., 2004). Indeed, mutation of residues within this helix can result in significant reduction in the ability of RyR to bind ryanodine and other ryanoids (Wang et al., 2003; Ranatunga et al., 2005). The enormous rates of permeant ion movement achieved in RyR indicate that the channel must have a large capture radius and, as a consequence, it is probable that the cytosolic cavity of the pore will be much wider than the constricted region within which TEA^+ blocks K^+ translocation, with the voltage drop across the channel extending some way into this cavity (Williams et al., 2001). Interactions within the voltage drop could contribute to the small differences in voltage dependence of interaction observed with ryanoids of differing formal charge (Tanna et al., 2000; 2003). The mode of ryanoid binding in the proposed cytosolic vestibule is under active investigation.

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Footnotes

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Legends to Scheme and Figures

Scheme 1: Proposed mechanism of TEA inhibition of RyR2 function. The scheme shows a representation of the pore of the RyR channel. The voltage drop across the pore is indicated towards the luminal end of the structure as a cross-hatched area and a ryanoid binding site is indicated by a circle. The position of this site is for convenience only and should not be interpreted as a statement about location in our published model (Welch et al (2004)). Ry indicates ryanoid and T^+ indicates TEA^+. The closed form of the pore (A) opens to a conducting form (B). The open pore can randomly bind ryanoid to form a partially conducting form of the open channel (C) or one TEA^+, at a site approximately 90% into the voltage drop normally involved in permeant cation translocation, to form a non-conducting state (D). TEA^+ interaction with the K^+ blocking site in the ryanoid-modified pore yields a non-conducting pore (E). Ryanoid binding alters pore geometry thus modifying both permeant cation conductance (Lindsay et al (1991)) and the binding of TEA^+ (Tanna et al (2001)). By First Law relationships, TEA^+ binding must also perturb ryanoid binding ($K_2K_4=K_3K_5$). TEA^+, a hydrophobic cation, also binds to the ryanoid-binding site preventing ryanoid association (F). Earlier quantitative structure-activity relationships have shown an amphipathic, anionic sub-site within the ryanodine binding site (Sutko et al (1997)). This scheme is consistent with the data presented in this communication and earlier studies (see text).

Figure 1 Time course of [^{3}H]-ryanodine binding in the absence (▲) and presence of 500 mM TEA^+ (Δ). Each point is the mean and SEM of 11-48 assays from 4-5 HSR

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preps. Solid lines are best fits to exponential association using the relationship: Ryanoid bound = B_i $(1 – \exp(-t/\tau))$, as described in the text.

Figure 2 The relationship between [^{3}H]-ryanodine binding and [TEA^+], the samples were incubated for 10 minutes. Each point is the mean ± SEM of 12-21 assays from 4-5 HSR preps. The solid line is the best fit to equation *1* obtained by non-linear regression with an IC_{50} of 668 mM.

Figure 3 Traces obtained from a single RyR channel in symmetrical 610 mM K^+ with 500 nM 21-amino-9α-hydroxyryanodine in the solution at the cytosolic side of the channel. The indicated concentrations of TEA^+ were added to both cytosolic and luminal solutions. Holding potential is +40 mV. O – open, C – closed and M - modified.

Figure 4 The relationship between probability of RyR channel modification by 21-amino-9α-hydroxyryanodine and [TEA^+]. P_{mod} is determined by monitoring dwell times in the unmodified and modified conductance states in 6 minute recordings with 500 nM 21-amino-9α-hydroxyryanodine in the solution at the cytosolic face of the channel and at a holding potential of +40 mV. Each point is the mean ± SEM of 4-18 experiments.

Figure 5 Variation in rates of association (♦) and dissociation (■) of cytosolic 21-amino-9α-hydroxyryanodine (500nM) with individual RyR channels held at +40mV at increasing concentrations of TEA^+. Each point is the mean ± SEM of 4-18

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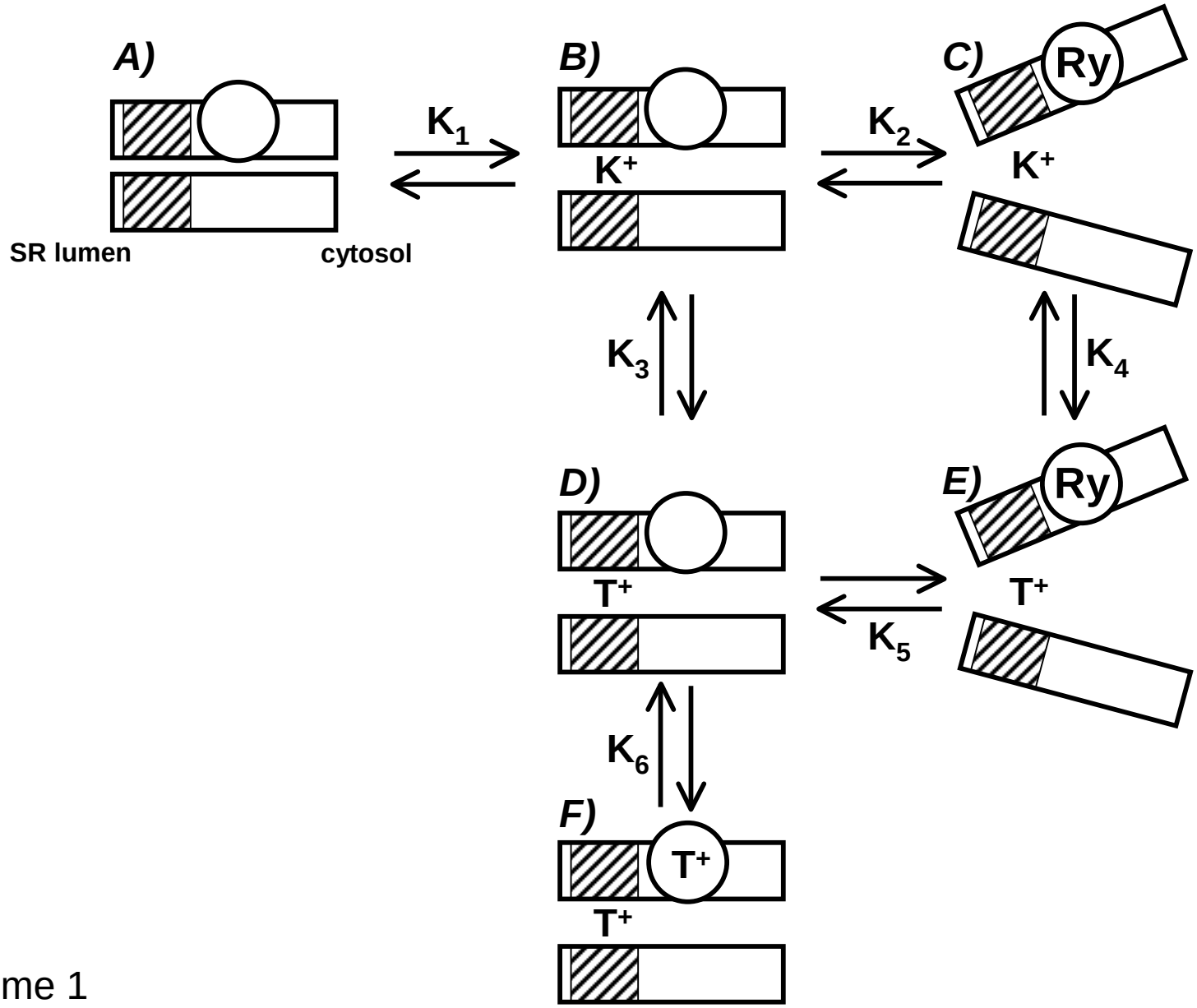
experiments. Lines drawn through the points were obtained by linear regression with parameters quoted in the text.

Figure 6 The influence of [TEA^+] on open channel unitary conductance (6A) and unitary conductance of the 21-amino-9α-hydroxyryanodine-modified state (6B). Each point is the mean of 4 - 18 experiments. The solid lines through the points were obtained by linear regression. Parameters derived from the plots are quoted in the text.

Figure 7 Analysis of the influence of [TEA^+] on the binding of [^3H]-ryanodine to RyR in isolated cardiac sarcoplasmic reticulum vesicles. Data presented in Figure 2 are re-plotted in log-log format. The solid line through the points was obtained by linear regression. Parameters derived from the plot are quoted in the text.



Scheme 1

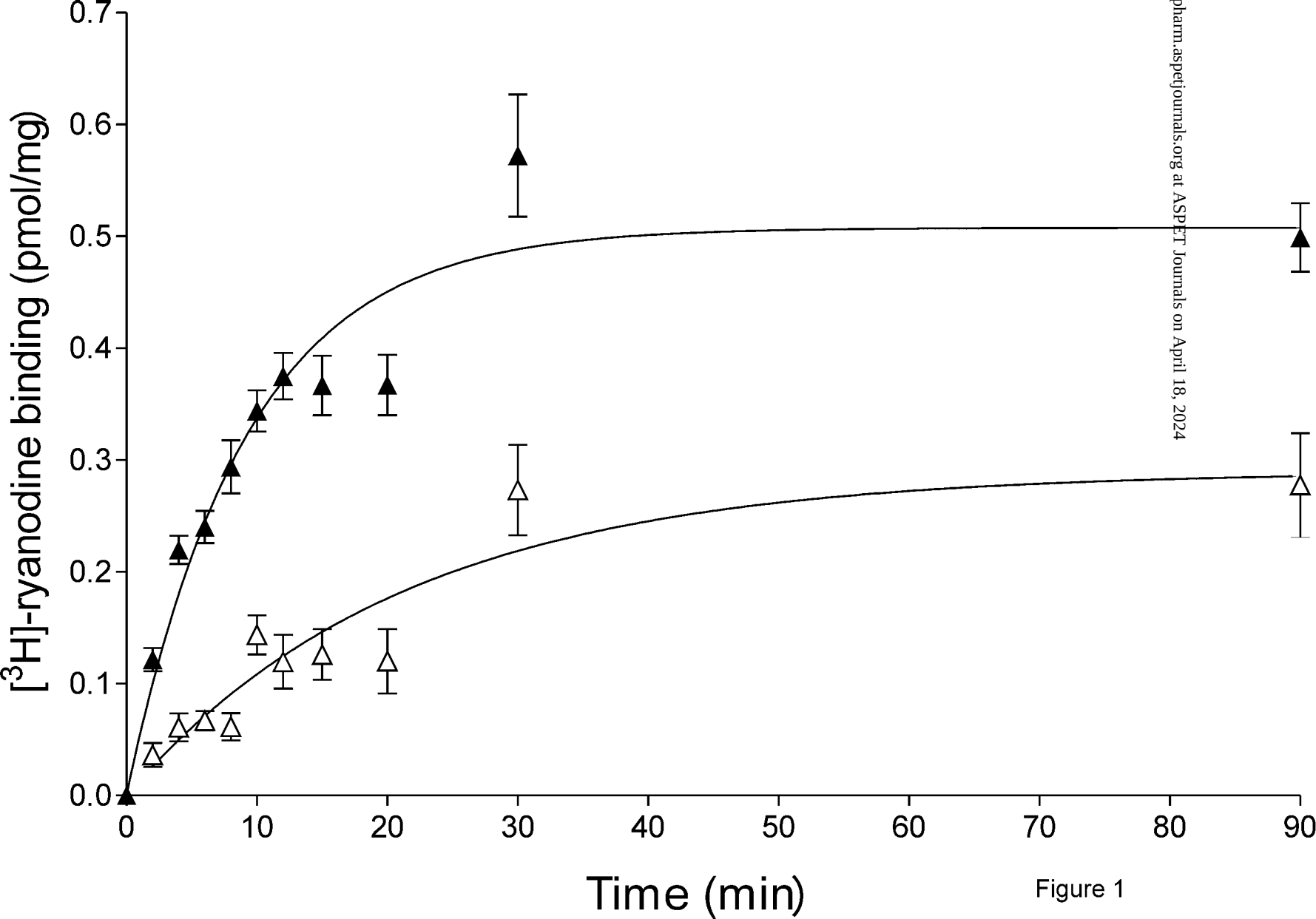


Figure 1

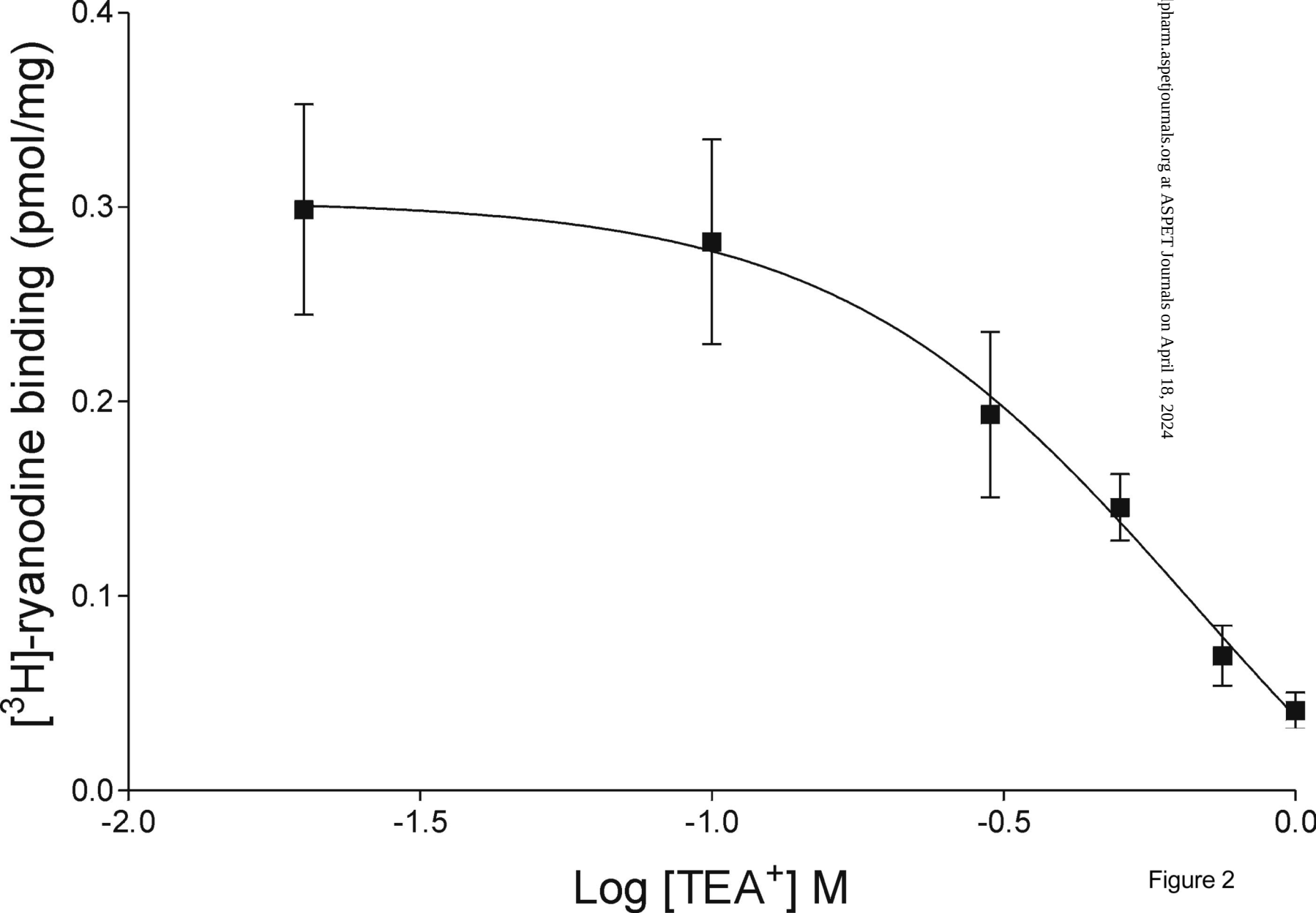


Figure 2

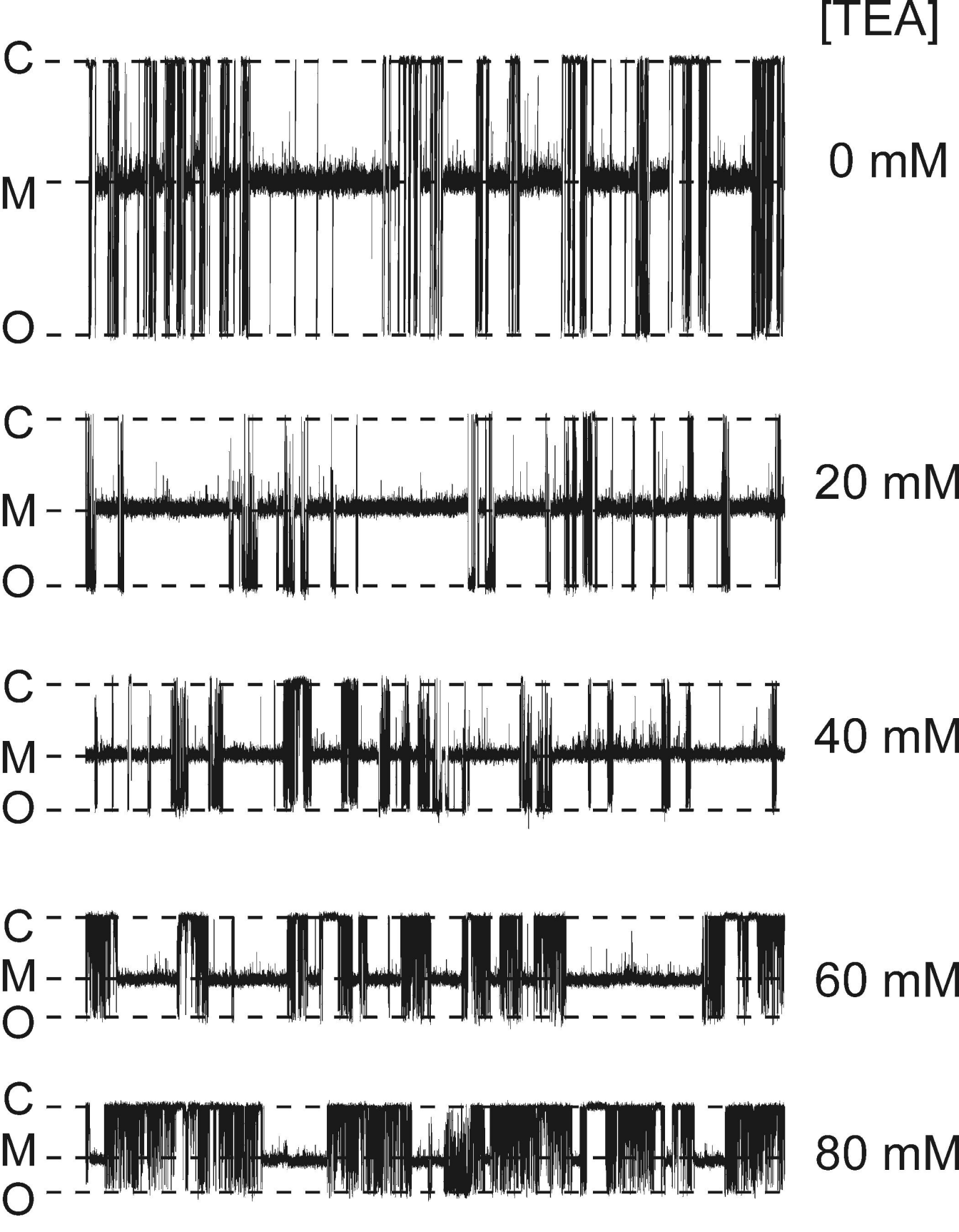


Figure 3

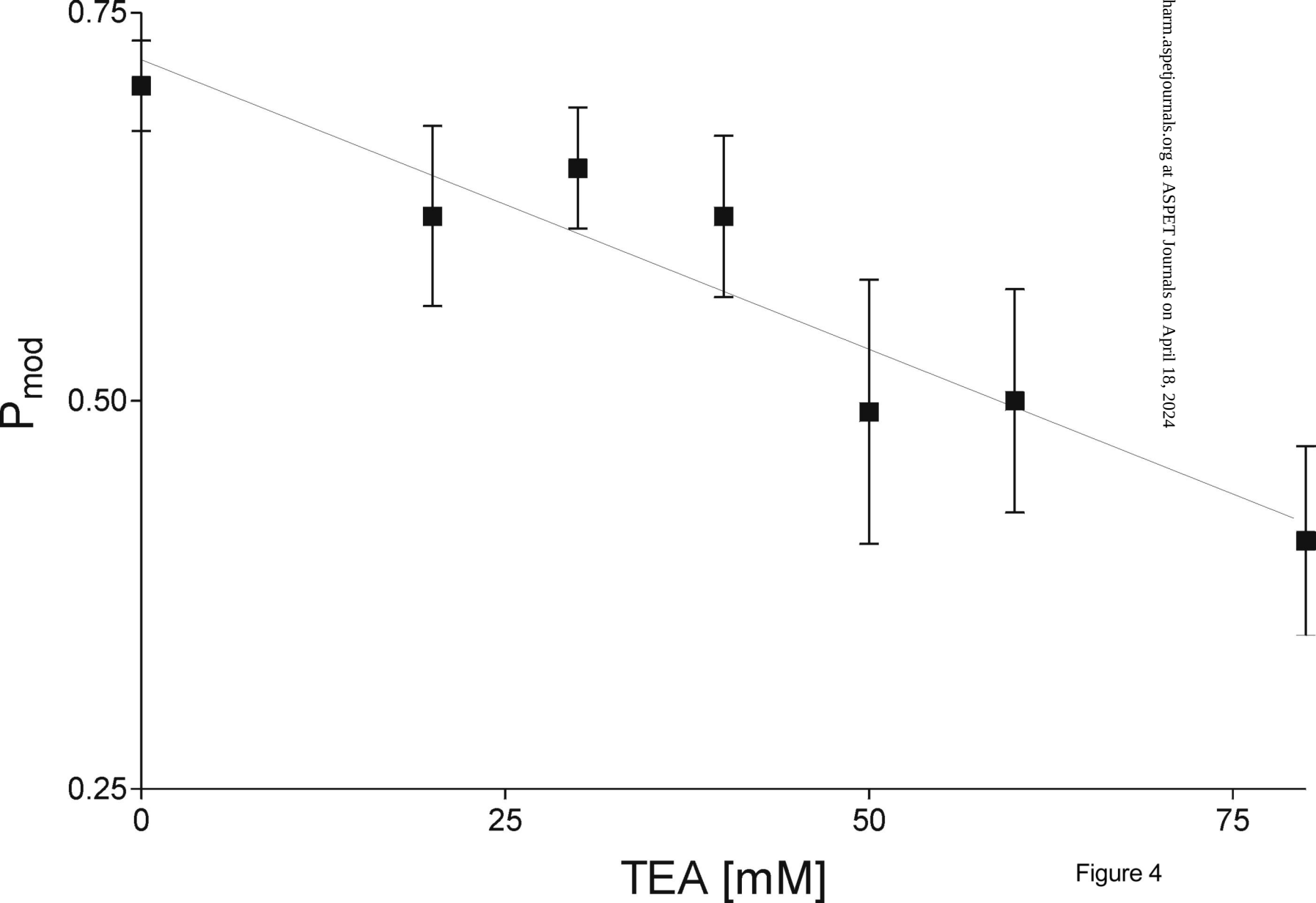


Figure 4

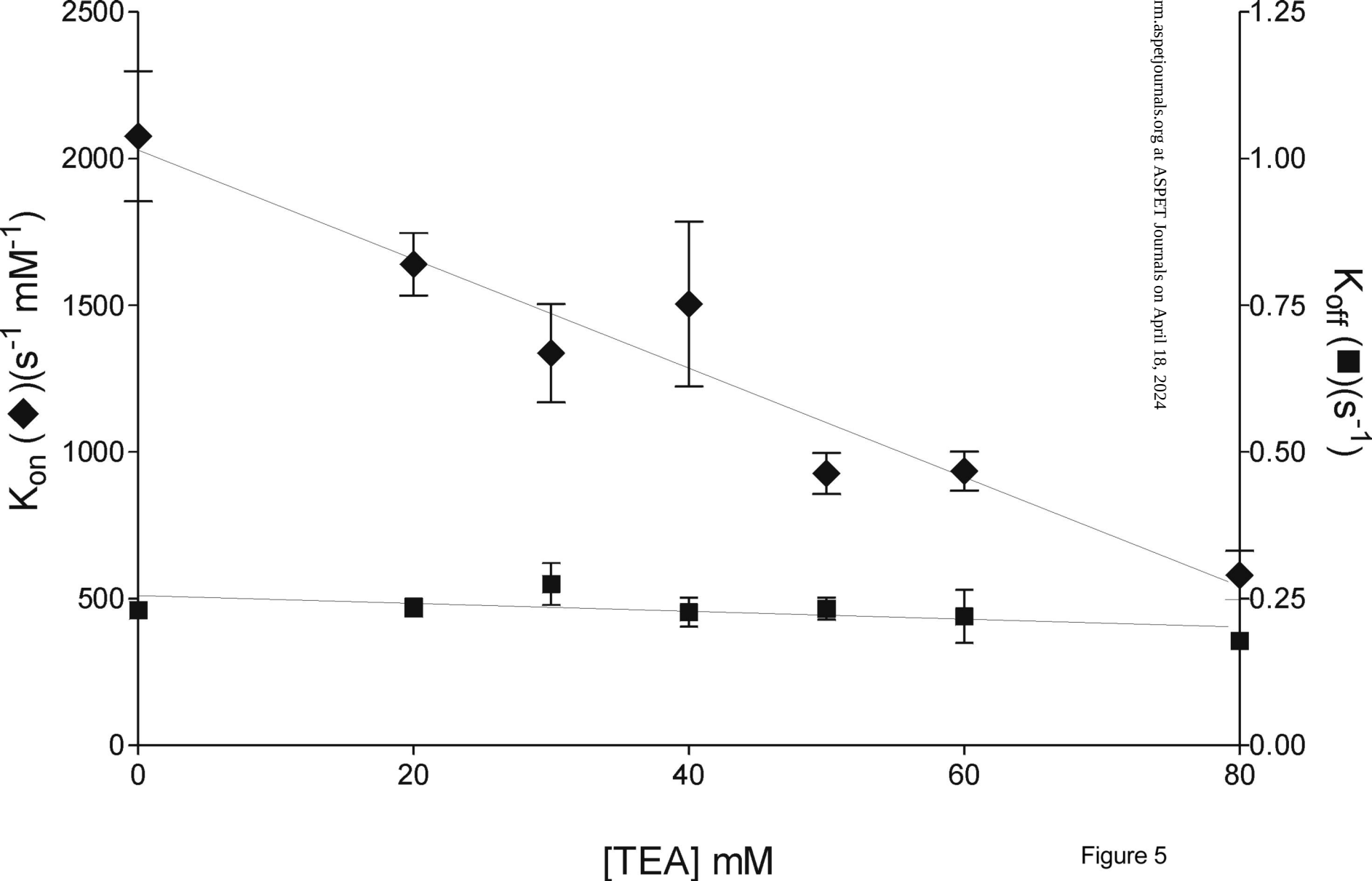
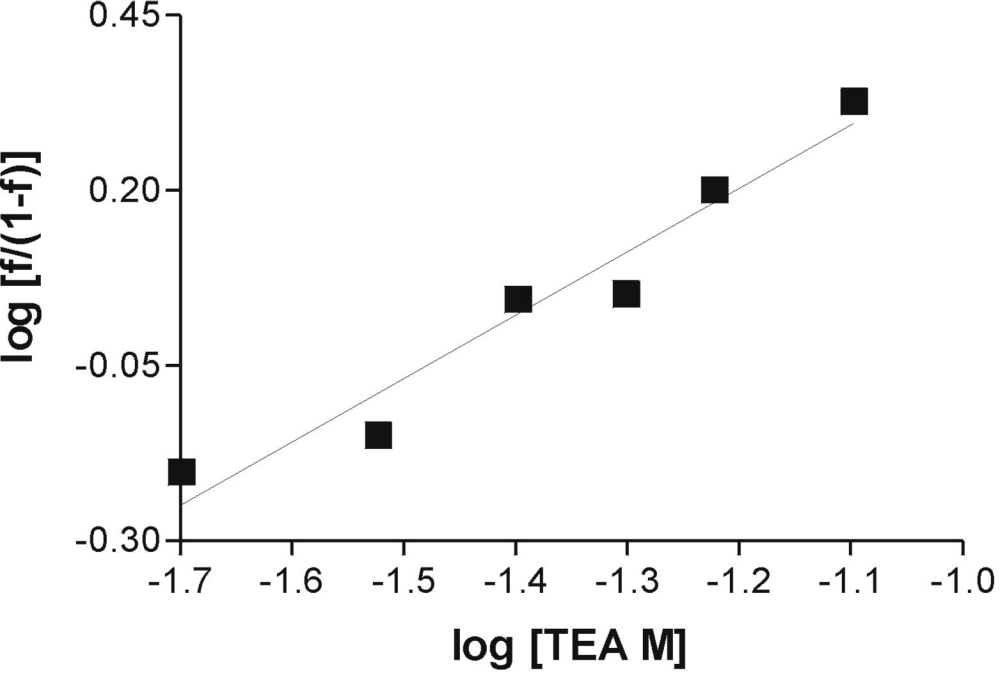


Figure 5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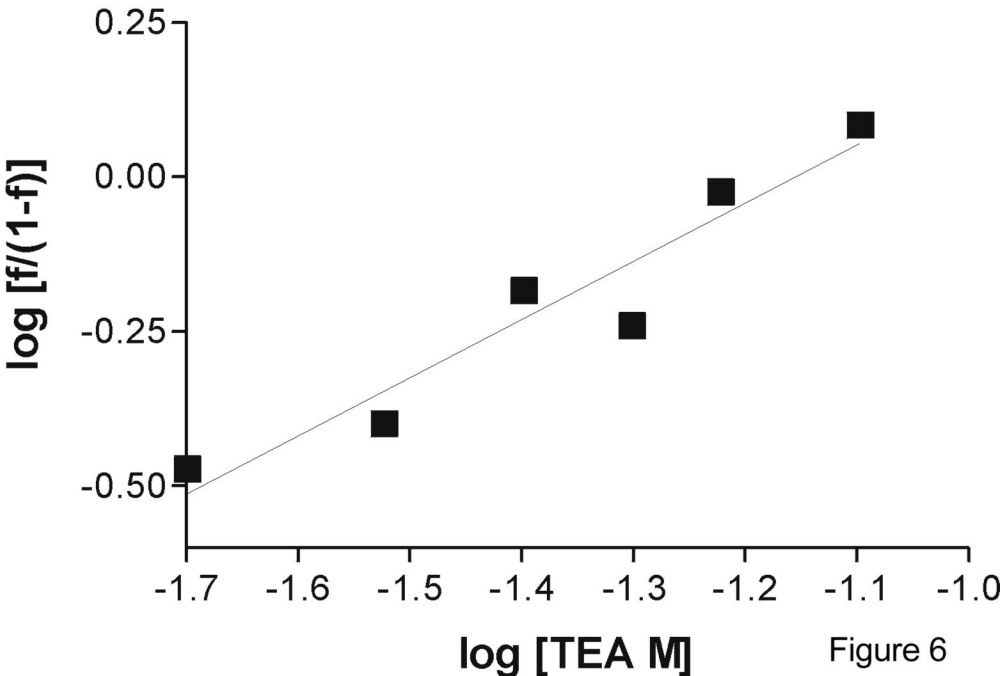
log [TEA M]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log [TEA M]

Figure 6

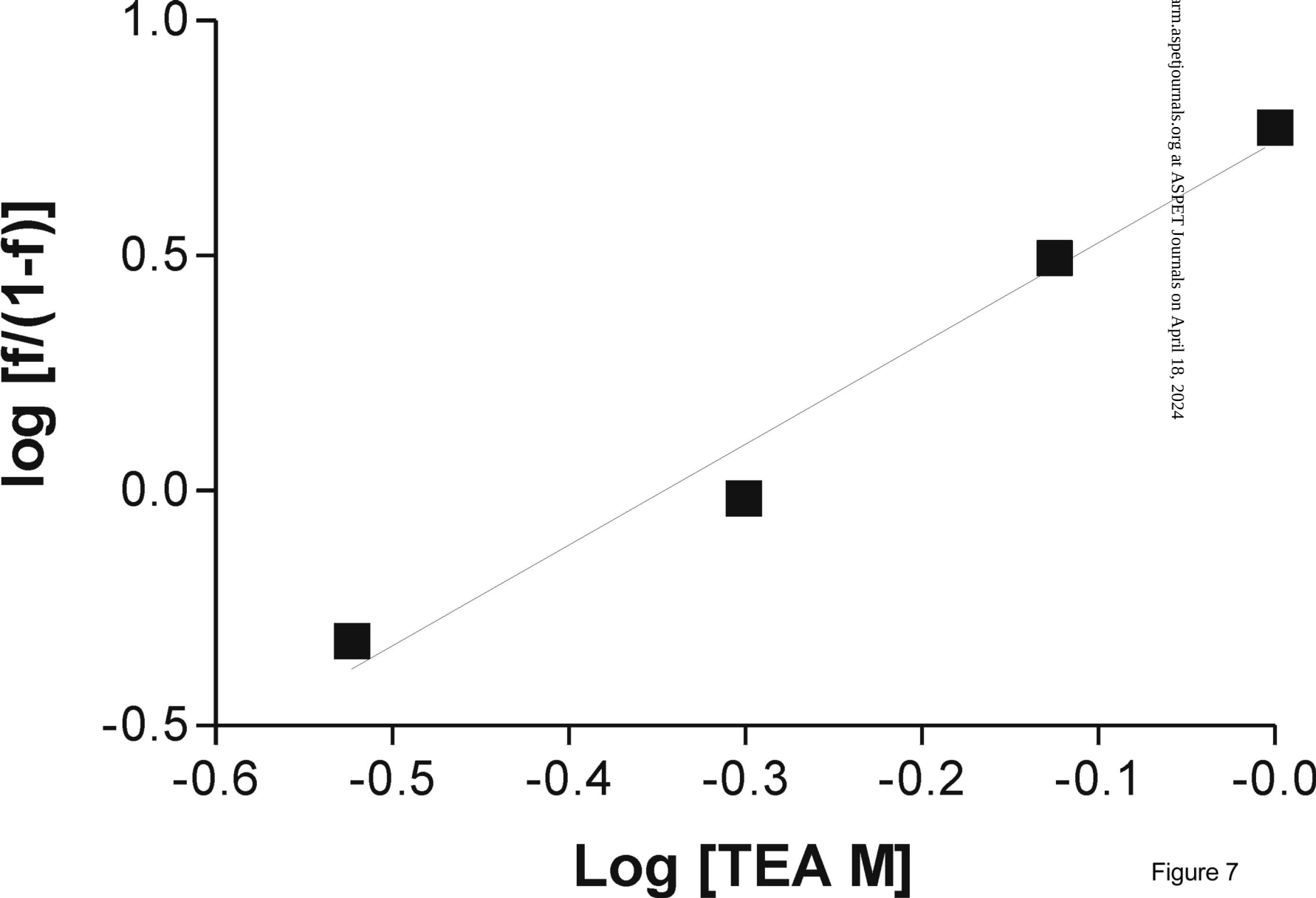

Figure 7