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The mycotoxin paxilline inhibits the cerebellar inositol 1,4,5-trisphosphate receptor

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Abstract

Paxilline, a tremorgenic alkaloid mycotoxin produced by *Penicillium paxilline*, is a reversible inhibitor of the cerebellar inositol 1,4,5-trisphophate ($InsP_3$) receptor. It inhibits the amount or extent of $InsP_3$-induced Ca^{2+} release (IICR), at sub-maximal concentrations of $InsP_3$, in a biphasic manner consistent with two inhibition constants (K_i's 6.7 and ≥ 400 μM). As paxilline does not affect $InsP_3$ binding to the receptor, it can be considered a non-competitive inhibitor. The fact that IICR is biphasic has been interpreted as there being two populations of $InsP_3$-sensitive Ca^{2+} stores, which release Ca^{2+} in either a fast or slow fashion. This study has shown that the rate constants for Ca^{2+} release from both the fast and slow populations are reduced by paxilline (100 μM) by about 70% and 60%, respectively. Detailed analysis of the way different concentrations of paxilline inhibit the rate constants for Ca^{2+} release indicates that the population of Ca^{2+} stores that contribute to the slower phase of Ca^{2+} release is more sensitive to the inhibitory action of paxilline.

Keywords: $InsP_3$ receptor; Ca^{2+} signalling; Paxilline; Mycotoxin

1. Introduction

The opening of the D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$) receptor and the subsequent increase in intracellular Ca^{2+} is crucial to many cellular processes, including fertilisation, metabolism and secretion (Berridge, 1993). The $InsP_3$ receptor is modulated by a number of different pharmacological compounds, which can be used to investigate its functional properties. Drugs capable of modulating or blocking $InsP_3$-induced Ca^{2+} release (IICR) could also have therapeutic potential, especially if it proves possible to target such drugs locally or to specific cell types (Wilcox et al., 1998; Michelangeli et al., 1995).

It has been previously noted that a variety of potassium-channel blockers, including quinine, 4-aminopyridine, 9-aminoacridine and tetraalkyl ammonium cations, can inhibit IICR (Michelangeli et al., 1995; Palade et al., 1989; Sayers and Michelangeli, 1993). Tetrahexyl ammonium cations are the most potent and are able to inhibit both the amount and the rate of Ca^{2+} release induced by $InsP_3$ from cerebellar microsomes at low μM concentrations (Palade et al., 1989; Sayers and Michelangeli, 1993). These studies also showed that blockade of K^+ channels by these drugs was not the underlying mechanism of IICR inhibition, as suggested by Muallem et al. (1985), since K^+ ionophores, including valinomycin, did not alleviate this inhibition.

It has been postulated that inhibition of IICR by tetraalkyl ammonium ions may result from interaction of the alkyl chains with hydrophobic regions of the Ca^{2+} channel, thereby preventing the flow of Ca^{2+} through the channel (Michelangeli et al., 1995). Inhibition of IICR by tetraalkyl ammonium cations was also shown not to be due to inhibition of $InsP_3$ binding to its receptor (Palade et al., 1989; Sayers and Michelangeli, 1993). Thus, these compounds act as "non-competitive" inhibitors of the $InsP_3$ receptor.

Paxilline, a mycotoxin produced by the fungus *Penicillium paxilline*, is a tremorgenic alkaloid that induces neurological disorders in vertebrate animals (Cole and Cox, 1981). It belongs to a family of tremorgenic alkaloids that include aflatrem, penitrem A, paspalinine, verruculogen and paspalicine. The ability of these compounds to induce

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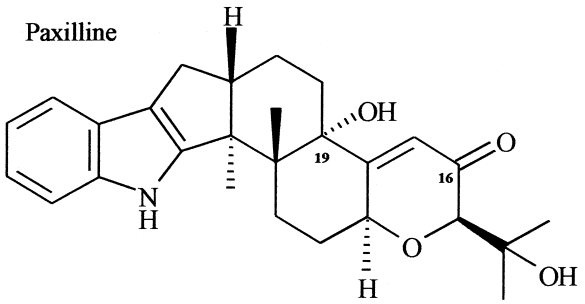

Fig. 1. The structure of paxilline.

tremors is critically dependent on a hydroxyl group at position 19 of the diterpene nucleus (see Fig. 1 for the structure of paxilline) (Knaus et al., 1994).Tremorgenic alkaloids such as paxilline are the most potent and selective nonpeptidyl inhibitors of high-conductance calcium-activated potassium channels, otherwise known as maxi-K channels (Knaus et al., 1994).

Here, we investigate the effects of the potassium channel inhibitor, paxilline, on the $InsP_3$ receptor and show that it inhibits IICR by reducing the rate constants for Ca^{2+} release.

2. Materials and methods

Fluo-3 was obtained from Sigma and paxilline obtained from Alomone Labs, $InsP_3$ was purchased from Alexis Corporation (UK) and [^{3}H]$InsP_3$ was obtained from Amersham Life Science. Paxilline was dissolved in dimethylsulfoxide (DMSO). DMSO was $\leq 0.25\%$ volume of any experiment, and was shown to have no effect on control experiments.

Cerebellar microsomes were prepared as described by Sayers et al. (1993). Briefly, approximately 40 rat cerebella were minced and homogenised (using a Teflon Potter–Elvehjem homogeniser) in 10 volumes of 5 mM Hepes, 0.32 M sucrose (pH 7.2) containing a cocktail of protease inhibitors (0.1 mM benzamidine, 0.1 mM phenylmethane suphonyl fluoride and 10 μM leupeptin). The homogenate was centrifuged at $500 \times g$ for 10 min. The resulting pellet was re-homogenised in 50 ml of the same buffer and centrifuged as before. The two supernatants were then pooled together and centrifuged for 20 min at $10{,}000 \times g$ in order to remove the mitochondria. The resulting supernatant was centrifuged for 1 h at $100{,}000 \times g$ to give the microsomal pellet, which was resuspended in the Hepes/sucrose buffer, snap frozen in liquid nitrogen and stored at -70°C until required.

Ca^{2+} uptake and release from cerebellar microsomes were measured by following the change in fluorescence of the Ca^{2+} indicator dye fluo-3 as described by Michelangeli (1991). Fluorescence changes were monitored in a Perkin-Elmer LS50B spectrofluorimeter, exciting at 506 nm and detecting emission at 526 nm, and were related to Ca^{2+} concentration using the following equation:

$$[Ca^{2+}] = K_d((F - F_{min})/(F_{max} - F))$$

where K_d is the dissociation constant for Ca^{2+} binding to fluo-3 (900 nM at 37°C, pH 7.2 in 100 mM K^+, Mezna and Michelangeli, 1995a), F is the fluorescence intensity of the sample and F_{min} and F_{max} are the fluorescence intensities of the sample in 1.25 mM EGTA and 1.5 mM $CaCl_2$, respectively.

Membranes (0.2 mg) were added to a stirred cuvette containing 2 ml of 40 mM Tris-phosphate, 100 mM KCl (pH 7.2) in the presence of 1.25 μM fluo-3, 10 μg/ml creatine kinase and 10 mM phosphocreatine. Ca^{2+} uptake was then initiated by the addition of 1.5 mM Mg-ATP. Once sufficient Ca^{2+} uptake had occurred, further uptake was inhibited by the addition of 0.25 mM sodium orthovanadate, which inhibits $> 90\%$ of Ca^{2+} pump activity. After the fluorescence had levelled off, indicating an equilibrium between Ca^{2+} efflux and influx, $InsP_3$ was then added. Total Ca^{2+} accumulation was measured by the addition of 12.5 μg/ml Ca^{2+} ionophore (A23187). The amount or extent of Ca^{2+} release induced by $InsP_3$ was expressed as a percentage of the Ca^{2+} mobilised by the ionophore.

Rapid measurements of IICR from cerebellar microsomes were carried out as described by Mezna and Michelangeli (1995b). Microsomal membranes, which had been loaded with Ca^{2+} and had their Ca^{2+} pumps inactivated by the addition of sodium orthovanadate, were introduced into the large syringe of a stopped-flow spectrofluorimeter (Applied Photophysics SX-17MV) and $InsP_3$ was added from a small syringe (drive ratio 10:1). The contents of the two syringes were rapidly mixed together and the changes in fluorescence recorded (monitored by excitation at 505 nm and detecting emission above 515 nm using a cut-off filter). The fluorescence data from the stopped-flow apparatus was compared with identical experiments undertaken on the conventional fluorimeter, such that the traces could be related to fractional Ca^{2+} release as described Mezna and Michelangeli (1995b). Typically the traces of between 6 and 10 experiments were averaged and the data were then analysed by non-linear least-squares fitting (Fig. P, Biosoft). Data were fitted to a biexponential equation assuming two independently occurring processes:

$$Ca^{2+} \text{ release} = A_1(1 - \exp^{-k_1 t}) + A_2(1 - \exp^{-k_2 t})$$

where A_1, A_2, k_1 and k_2 are the amplitudes and rate constants of fractional Ca^{2+} release for the fast and slow components, respectively, and t is time (s). The goodness-of-fit of each data set using this equation was assessed by determination of the χ^2 value and which was less than 0.1 in all cases. The fluorescence changes when related to Ca^{2+} concentration were around the K_d value for Ca^{2+} binding to fluo-3. Consequently, over the Ca^{2+} concentra-

tions being measured, fluorescence change was linearly related to Ca^{2+} concentration (Mezna and Michelangeli, 1995a, 1996).

The binding of [^{3}H]$InsP_3$ to microsomal membranes was measured as described in Sayers et al. (1993). Approximately 0.3 mg of microsomal membranes were suspended in 0.5 ml of binding buffer (50 mM Tris/HCl, pH 8.3, 1 mM EDTA, 100 mM KCl) doped with 8 nCi/ml [^{3}H]$InsP_3$. Specific binding was measured at 40 nM $InsP_3$ (the K_d value for $InsP_3$ binding to cerebellar microsomes under our experimental conditions). Non-specific binding was measured in the presence of 10 μM excess non-radioactive $InsP_3$. Following the addition of microsomal membranes, the assay mixture was incubated on ice for 10 min. Bound [^{3}H]$InsP_3$ was then separated from free [^{3}H]$InsP_3$ by centrifugation of the samples at 18,000 × g for 20 min at 4°C. The resulting supernatant was removed and the pellet was washed with distilled water three times. After air drying for approximately 1 h, the pellets were solubilised in 150 μl of solvable tissue solubiliser (Dupont) and incubated for 3 h at 37°C. Ultima flow scintillant (ammonium formate) was added to each sample to give a final volume of 1.5 ml. The radioactivity present in each sample was then determined using liquid scintillation spectrometry, counting for 5 min per sample. Specific [^{3}H]$InsP_3$ binding was expressed as pmol [^{3}H]$InsP_3$ bound per mg of microsomal protein.

3. Results

Fig. 2A illustrates the effect of increasing concentrations of paxilline on the extent or amount of IICR (expressed as a percentage of that releasable by A23187), induced by half-maximal (0.33 μM) and maximal (20 μM) $InsP_3$ concentrations. Cerebellar microsomes were incubated with the alkaloid for 5 min prior to the addition of $InsP_3$. It was determined that this period of time was sufficient for paxilline to have its maximum effect. Paxilline inhibition of the half-maximal extent of IICR was biphasic in nature, with one apparent inhibition constant of 6.65 ± 1.85 μM (standard deviation S.D., $n = 3$) and a second one of ≥ 400 μM. Paxilline only had a small influence on the maximal extent of IICR induced by 20 μM $InsP_3$.

Fig. 2B shows the effect of different concentrations of paxilline on the extent of IICR as a function of [$InsP_3$]. The maximum extent of IICR observed with 20 μM $InsP_3$ was only slightly reduced, from 29.1 ± 2.6% to 26.6 ± 1.8%, (S.D., $n = 3$) in the presence of 100 μM paxilline; however, this was statistically significant ($p < 0.02$). The concentration of $InsP_3$ required to cause half-maximal release of Ca^{2+} was increased from 0.33 ± 0.05 μM, in the absence of the inhibitor, to 0.63 ± 0.08 μM (S.D., $n = 3$) in the presence of 100 μM paxilline, which was again statistically significant ($p < 0.002$).

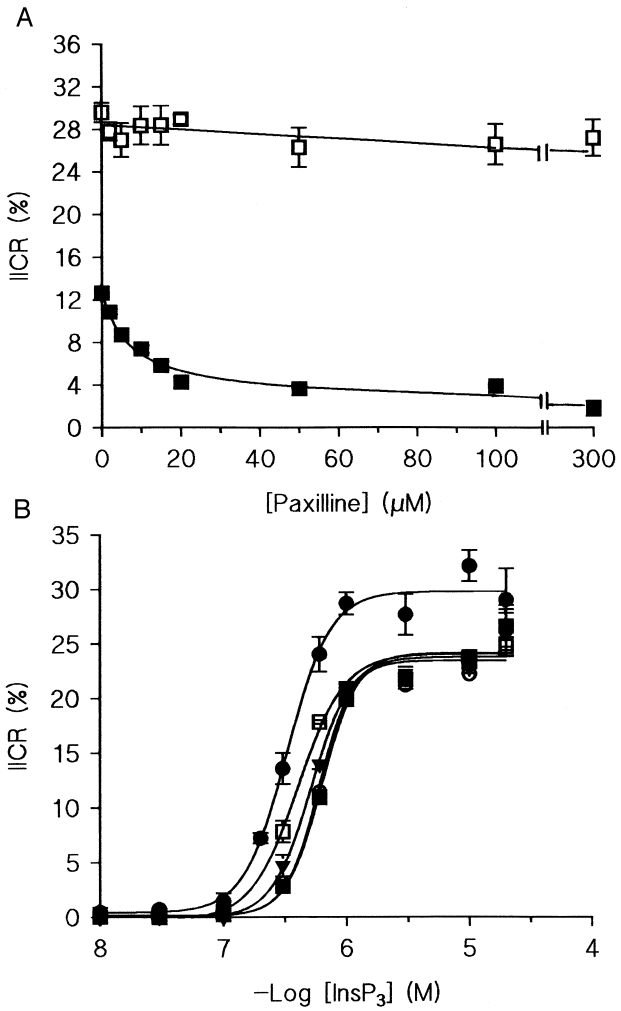

Fig. 2. The effects of paxilline on the extent of IICR. Panel (A) shows the effect of paxilline on the extent of IICR induced by half-maximal (0.33 μM, solid squares) and maximal (20 μM, open squares) $InsP_3$ concentrations. The extent of release is expressed as a percentage of the amount releasable by ionophore. Panel (B) shows the effect of 5 μM (open squares), 10 μM (solid inverted triangles), 50 μM (open circles) and 100 μM (solid squares) paxilline on quantal IICR, compared to control (solid circle). Cerebellar microsomes were incubated with paxilline for 5 min prior to the addition of $InsP_3$ at a number of concentrations. Each data point represents the mean ± S.D. of three separate determinations.

Paxilline is a reversible inhibitor of the $InsP_3R$ since when cerebellar microsomes were pre-incubated with 20 μM paxilline for 5 min and then diluted to 1 μM before Ca^{2+} loading and IICR was undertaken, addition of half-maximal (0.33 μM) and maximal (20 μM) doses of $InsP_3$ resulted in control levels of Ca^{2+} release.

[^{3}H]$InsP_3$ binding studies were undertaken in order to assess whether paxilline was a competitive inhibitor able to displace $InsP_3$ from the receptor. Table 1 shows that the binding of [^{3}H]$InsP_3$ to rat cerebellar microsomes when measured at 40 nM $InsP_3$, which is the Kd for $InsP_3$ binding under our experimental conditions, was unaffected by paxilline at concentrations up to 100 μM.

Table 1
Effects of paxilline on [^{3}H]$InsP_3$ binding to cerebellar microsomes

Paxilline (μM)	Specific [^{3}H]$InsP_3$ bound (pmol/mg)
0	7.32 ± 0.05
10	7.46 ± 0.25
20	6.93 ± 0.19
50	7.22 ± 0.17
100	7.20 ± 0.20

The effects of paxilline on $InsP_3$ binding was undertaken in the presence of 40 nM [^{3}H]$InsP_3$, pH 8.3 at 4°C. Non-specific binding was undertaken in the presence of 10 μM excess non-radioactive $InsP_3$. The values represent the mean ± S.D. of between three and five determinations.

As we have previously shown, the time course of IICR from cerebellar microsomes is biphasic in nature, as this release best fits a biexponential process (Mezna and Michelangeli, 1995a,b, 1996, 1998). We have suggested that this arises from heterogeneity within $InsP_3$-sensitive Ca^{2+} stores and have interpreted this behavior as coming from two types of Ca^{2+} stores which release their Ca^{2+} in response $InsP_3$ at either a rapid rate (fast component) and a slower rate (slow component) (Mezna and Michelangeli, 1995a,b, 1996, 1998). The effects of increasing concentrations of paxilline on the time course of IICR, at 20 μM $InsP_3$, were studied and were shown to fit very well to the biexponential equation. The effects of both 5 μM and 50 μM paxilline are illustrated in Fig. 3A.

The rate constants and amplitudes for the two components of IICR generated from all of the time course traces are shown in Fig. 3B and C, respectively. The amplitude of the fast component was unaffected whilst the amplitude of the slow component was slightly reduced, from 0.30 in the absence of paxilline to 0.2 in the presence of 100 μM paxilline. Both of the rate constants decreased in response to increasing concentrations of paxilline. The rate constant for the fast component decreased from 1.09 ± 0.05 s^{-1} in the absence of paxilline to 0.36 ± 0.05 s^{-1} in the presence of 100 μM paxilline. The rate constant for the slow component, meanwhile, decreased from 0.28 ± 0.02 to 0.12 ± 0.02 s^{-1} (S.D., $n = 6–10$) in the presence of 100

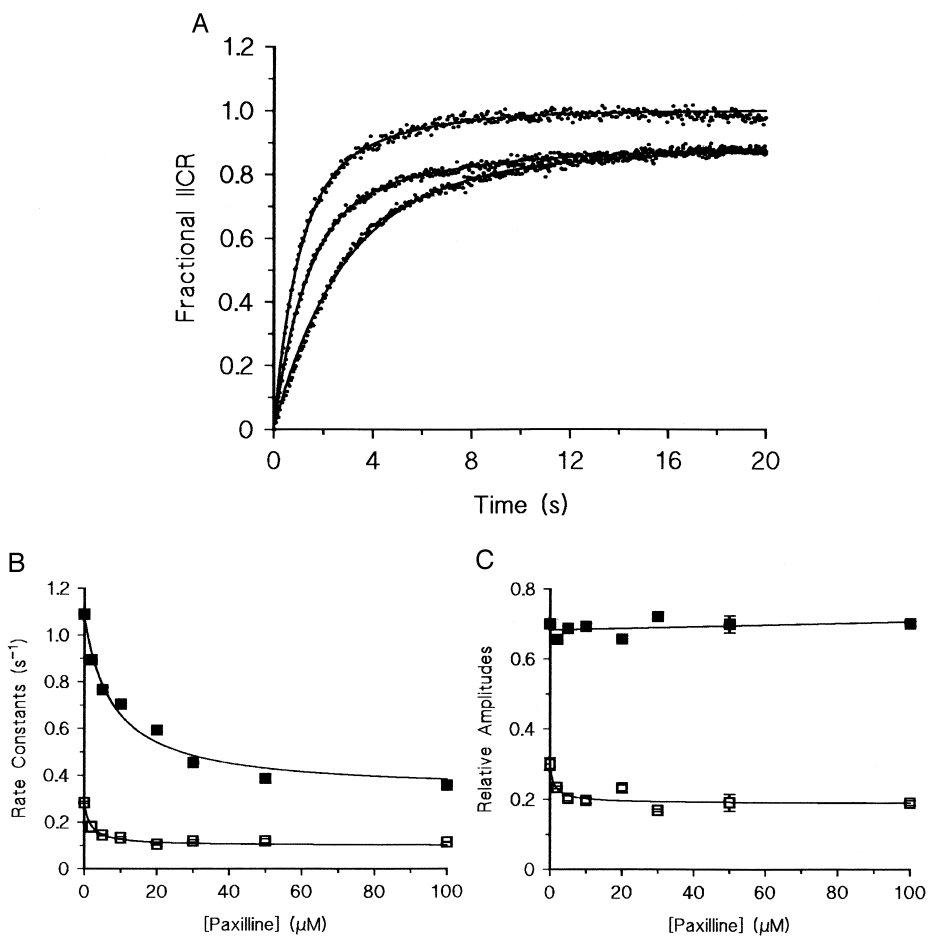

Fig. 3. The effects of paxilline on the kinetics of IICR. Panel (A) shows the effect of paxilline on the transient kinetics of IICR. IICR was measured using an $InsP_3$ concentration of 20 μM, in the presence of 0, 5, and 50 μM paxilline (from top to bottom). Each trace represents the mean of between 6 and 10 sequential acquisitions. The solid lines through the data points represent the best fit using the biexponential equation. In all cases, the goodness-of-fit as determined by the χ^2 values was less than 0.1. Panels (B) and (C) illustrate the effect of paxilline on the rate constants and relative amplitudes of Ca^{2+} release for the fast (solid squares) and slow components (open squares) of IICR, at a fixed $InsP_3$ concentration of 20 μM. The error bars represent the mean ± S.D. of the calculated rate constants and amplitudes which give the best fit to the experimental data.

μM paxilline, corresponding to decreases in the rate constants of about 70% and 60% for the fast and slow phases, respectively. The apparent dissociation constants calculated from the inhibition of the rate constants for the two components at a variety of paxilline concentrations were significantly different—7.92 ± 1.22 and 1.70 ± 0.30 μM for the fast and slow components, respectively ($p < 0.005$). Therefore, it appears that paxilline has a greater effect on the slow phase component of Ca^{2+} release.

4. Discussion

We have shown that paxilline acts as an inhibitor of IICR from rat cerebellar microsomes. It inhibited the half-maximal extent of IICR in a biphasic manner, with one apparent K_i of 6.65 ± 1.85 μM and a second apparent K_i of ≥ 400 μM. This suggests that there are possibly two distinct binding sites for paxilline on the $InsP_3$ receptor, which have different affinities. Whilst paxilline inhibited the extent of IICR at sub-maximal $InsP_3$ concentrations (0.33 μM), it had only a small effect on the extent of IICR at 20 μM $InsP_3$ Paxilline inhibition is reversible, since we have shown that inhibition by this compound can be reversed by dilution. Thus, paxilline must form a dynamic complex with the $InsP_3$ receptor resulting in altered receptor properties.

Paxilline had no effect on [^{3}H]$InsP_3$ binding, suggesting that it does not compete with $InsP_3$ or modify the $InsP_3$ binding site. Other K^+ channel blockers that inhibit IICR have also been shown to have no effect on [^{3}H]$InsP_3$ binding (Palade et al., 1989; Sayers and Michelangeli, 1993). Consequently, paxilline is a "non-competitive inhibitor" of the $InsP_3$ receptor, even though it was shown to have little affect on the amount of releasable Ca^{2+} at maximal $InsP_3$ concentrations. In a review on pharmacological modulators of the $InsP_3$ receptors (Michelangeli et al., 1995), inhibitors which reduce $InsP_3$-induced Ca^{2+} release but do not affect $InsP_3$ binding are classified as "non-competitive" inhibitors to distinguish them from competitive inhibitors like heparin, which do affect binding.

The rate constants for Ca^{2+} release were significantly reduced by paxilline at maximal $InsP_3$ concentrations. Similarly it has been shown in other studies that the rate of Ca^{2+} release is independent of the extent of Ca^{2+} release (Mezna and Michelangeli, 1995a,b, 1996, 1998). This suggests that these two aspects of IICR are distinct processes. Transient kinetic studies showed that IICR from rat cerebellar microsomes is a biexponential process, with fast and slow components. Biphasic release is thought to result from the existence of heterogeneous $InsP_3$-sensitive Ca^{2+} stores (Mezna and Michelangeli, 1995b; Hirose and Iino, 1994), because the sensitivity of the rate constants for the two phases for $InsP_3$ is different. The simplest explanation for such an observation is to assume that there are at least two populations/pools of Ca^{2+} stores which independently release their Ca^{2+}, in response to $InsP_3$, in either a fast or a slow manner (Mezna and Michelangeli, 1995b, 1996; Hirose and Iino, 1994). Thus, both components of Ca^{2+} release are due to a distinct population of Ca^{2+} stores, which have functionally different $InsP_3$ receptors. Heterogeneity of the $InsP_3$-sensitive Ca^{2+} stores could be accounted for by the presence of different receptor subtypes and receptor density, the differential expression of splice variants, the presence of modulatory proteins or the phosphorylation state of the receptors. More recently, an alternative mechanism to explain the biphasic behaviour of IICR has been proposed (Marchant et al., 1997; Marchant and Taylor, 1998). These studies have suggested that biphasic IICR arises from a single population of $InsP_3$-sensitive Ca^{2+} stores, in which the $InsP_3$-sensitive Ca^{2+} channel is initially opened by $InsP_3$ and then followed by a time-dependent partial inactivation of the channel. However, such a mechanism seems unlikely, since we have shown that the way in which the rate constants for the two phases change with [$InsP_3$] are dissimilar and therefore there is heterogeneity in the sensitivity to $InsP_3$ for the two phases (Mezna and Michelangeli, 1995b, 1996), which would be unlikely if the two phases were occurring through the same Ca^{2+} channel.

In the presence of paxilline, the time course of IICR from rat cerebellar microsomes remained biexponential in nature, demonstrating that paxilline did not operate by completely knocking out one of the components of Ca^{2+} release. The amplitudes of both the fast and slow components were little affected. However, both of the rate constants decreased in response to increasing concentrations of paxilline, suggesting that both types of Ca^{2+} pool were affected by paxilline. The rate constant for the fast component decreased from 1.09 s^{-1} in the absence of paxilline to 0.36 s^{-1} in the presence of 100 μM paxilline. The rate constant for the slow component, meanwhile, decreased from 0.28 s^{-1} to 0.12 s^{-1} in the presence of 100 μM paxilline.

The fact that this inhibitor (and others, Mezna and Michelangeli, 1998) differentially affects the rate constants of IICR for the two phases would argue strongly that in our case these phases arise from distinct Ca^{2+} stores, which release their Ca^{2+} differently and independently from each other. As such, this would preclude the possibility that the two phases arise through a single population of $InsP_3$-sensitive Ca^{2+} channel, which is initially activated then partially inactivated by $InsP_3$, as proposed in Marchant et al., 1997 and Marchant and Taylor, 1998.

Xestospongins, a group of macrocyclic bis-1-oxaquinolizidines isolated from sponges of the *Xestospongia* species, have been shown to inhibit IICR in cerebellar microsomes. They have been shown to act as non-competitive antagonists, and have been proposed to block Ca^{2+} release by either allosterically uncoupling ligand binding from Ca^{2+} release or by sterically blocking the channel

pore (Gafni et al., 1997). Tetraalkyl ammonium cations are also proposed to inhibit IICR by blocking the Ca^{2+} conducting pore of the $InsP_3$ (Sayers and Michelangeli, 1993). Paxilline may thus inhibit IICR in one of these ways.

From this and earlier studies, it is now apparent that the term "non-competitive" inhibitor for the $InsP_3$ receptor encompasses a number of different groups of agents which although do not affect $InsP_3$ binding, can alter IICR in a variety of ways. For instance, ethanol inhibits the extent of IICR without affecting the rate constants for Ca^{2+} release (Mezna et al., 1996), while acylphosphonates have little effect the extent of IICR, but reduce the rate constants only for the fast phase of release (Mezna and Michelangeli, 1998). Now in this study, we have identified another subtype of non-competitive inhibitor in which both the extent of IICR is inhibited as are the rate constants of both phases of release, but particularly the slow phase. The underlying mechanism of these different types of "non-competitive" inhibition will need to be resolved and in doing so may also give us new insights into the molecular functioning of this complex channel.

The pharmacology of the $InsP_3$-receptor (which is a K^+-activated Ca^{2+} channel, Mezna and Michelangeli, 1995a) and the maxi-K channel (which is a Ca^{2+}-activated K^+ channel, Knaus et al., 1994) is quite striking, since there are a number of quite distinct pharmacological agents which inhibit both (i.e., paxilline, tetraalkyl ammonium and ethanol, Sayers and Michelangeli, 1993; Knaus et al., 1994; Mezna et al., 1996; Cai et al., 1998; Walters et al., 2000). The maxi-K channels consist of two subunits, the α subunit being the ion channel and the β subunit having a modulatory function. Studies have shown that paxilline binds to the α subunit with an affinity in the low nM range (Sanchez and McManus, 1996). This subunit appears to have a number of regions of amino acid sequence similarity as assessed using the "BestFit" algorithm found within the "Genetics Computer Group" program package (GCG, supplied by the University of Wisconsin) for identifying regions of similarity between two protein sequences. The first region is between amino acids 420–468 of the α subunit of human maxi-K channel, corresponding to a region between hydrophobic segments S6 and S7 (Adelman et al., 1992), and amino acids 2090–2138 within the modulatory domain of the type I $InsP_3$ receptor (Mignery et al., 1990). These regions have a 45% sequence similarity. The other is located at the amino terminus of the maxi-K channel (amino acids 5–43) and around the putative ATP binding site of the rat $InsP_3$ receptor (amino acids 1767–1805), which have 36% sequence similarity. One might, therefore, speculate that the putative binding site for these drugs may be at such regions of high sequence similarity.

In conclusion, paxilline is a novel "non-competitive" inhibitor of the $InsP_3$ receptor present in rat cerebellar microsomes. Paxilline reduces both rates of Ca^{2+} release. Of the two populations of $InsP_3$-sensitive stores, which either release Ca^{2+} in a fast or slow fashion, it seems as though the population which releases Ca^{2+} more slowly is more sensitive to the inhibitory action of paxilline. In addition, the fact that this unusual compound inhibits both $InsP_3$ receptors as well as K^+ channels, reinforces the similarity (at least in their pharmacology) between these two types of ion channels.

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