Inhibition of the cerebellar inositol 1,4,5-trisphosphate-sensitive Ca^{2+} channel by ethanol and other aliphatic alcohols

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The effects of ethanol and other aliphatic alcohols on the endoplasmic reticulum Ca^{2+} pump and the inositol 1,4,5-trisphosphate (InsP_3)-sensitive Ca^{2+} channel were studied in pig cerebellar microsomes. Methanol, ethanol and propanol all stimulated ATP-dependent Ca^{2+} uptake, whereas butanol inhibited this process. Ethanol inhibited InsP_3 -induced Ca^{2+} release [half-maximal inhibition at 3.5%, v/v (600 mM)]. However, ethanol affected only the amount of InsP_3 -releasable Ca^{2+} , without affecting the concentration of InsP_3 required to induce half-maximal release. Other alcohols of longer chain length were

more potent than ethanol at inhibiting InsP_3 -induced Ca^{2+} release, but none of the alcohols tested affected [^3H] InsP_3 binding to its receptor. Using stopped-flow techniques, measurements of the rate of InsP_3 -induced Ca^{2+} release in the preparation of pig cerebellar microsomes used in this study showed the kinetics to be monophasic, with a rate constant of 0.93 s^{-1} at $20\text{ }\mu\text{M}$ InsP_3 . This rate constant was dependent upon InsP_3 concentration, decreasing to 0.38 s^{-1} at $0.25\text{ }\mu\text{M}$ InsP_3 . Ethanol was shown to reduce the fractional amount of InsP_3 -induced Ca^{2+} release without significantly affecting the rate constant for this process.

INTRODUCTION

Inositol 1,4,5-trisphosphate (InsP_3) stimulates Ca^{2+} release, usually from the endoplasmic reticulum by opening an InsP_3 -sensitive Ca^{2+} channel (InsP_3 receptor), in a process known as InsP_3 -induced Ca^{2+} release (IICR) [1]. As the cerebellum was found to be particularly abundant in InsP_3 receptors [2,3], cerebellar microsomes have been an extensively used system in the study of this protein.

The receptor consists of a single polypeptide chain of approx. 300 kDa and is believed to contain six transmembrane helices at the C-terminal region, which constitutes the channel domain, while the InsP_3 binding domain is located close to the N-terminus. These two domains are linked together by a modulatory domain which, in the type 1 isoform (the isoform predominantly located in the cerebellum [4]), contains two putative ATP binding sites, protein kinase A-dependent phosphorylation sites and Ca^{2+} binding sites [5–7].

One of the most striking features of this Ca^{2+} channel is that the Ca^{2+} release process appears ‘quantal’ in nature: following addition of submaximal doses of InsP_3 a fraction of Ca^{2+} in the stores is released, and more Ca^{2+} can only be released after addition of higher concentrations of InsP_3 [8]. Several theoretical models have been proposed to account for such behaviour. One model suggests that Ca^{2+} stores discharge their contents in an ‘all-or-nothing’ fashion and that stores are heterogeneous in their sensitivities to InsP_3 [8,9]. Alternative models suggest that the quantal phenomenon could be explained by the presence of more homogeneous Ca^{2+} stores which partially discharge their contents in response to different concentrations of InsP_3 and which may depend upon luminal Ca^{2+} concentrations [10–13].

Numerous studies have shown that IICR is affected by variety of compounds [13]. The K^+ channel blocker tetrahexyl ammonium is an extremely potent inhibitor ($K_i\text{ }3\text{ }\mu\text{M}$) which decreases both the amount and the rate of IICR but does not affect either the concentration of InsP_3 required to cause half-maximal release (IC_{50}) or [^3H] InsP_3 binding to its receptor [14]. Caffeine, another inhibitor of IICR, on the other hand, shifts the IC_{50} value such that more InsP_3 is required to release Ca^{2+} , but

does not affect either the total Ca^{2+} release at higher InsP_3 concentrations or InsP_3 binding [15]. Heparin also inhibits IICR by binding with high affinity to the InsP_3 binding domain of the receptor, hence increasing the concentrations of InsP_3 required for half-maximal Ca^{2+} release and decreasing InsP_3 binding [16]. More recently we have shown that physiological concentrations of polyamines inhibit IICR [17]. For example, spermine inhibited IICR by both increasing the IC_{50} value and decreasing the maximal amount of Ca^{2+} release, without affecting ligand binding. As highlighted here, a number of unrelated inhibitors of IICR must be affecting the mechanism of the InsP_3 receptor in different ways, and as such may help in the elucidation of the operation of this channel.

Ilyin and Parker [18] have recently reported that alcohols have diverse effects on the InsP_3 signalling processes in *Xenopus* oocytes. By indirectly monitoring Ca^{2+} mobilization by measuring Ca^{2+} -sensitive chloride currents, methanol was shown to have a negligible effect on Ca^{2+} release, while ethanol could both stimulate and inhibit Ca^{2+} release depending on the InsP_3 concentrations used. Longer-chain alcohols, however, showed only inhibition.

Here we examine the effects of ethanol and other aliphatic alcohols on the InsP_3 -sensitive Ca^{2+} channel from pig cerebellar microsomes, by studying their effects on the kinetics of IICR. We also examine the effects of alcohols on InsP_3 binding to microsomes and assess their influence upon the endoplasmic reticulum Ca^{2+} pump.

MATERIALS AND METHODS

Pig brains were obtained from a local abattoir and cerebellar microsomes were prepared as described in [19], with all steps carried out at 4°C . Whole pig cerebellum was minced and homogenized using a Teflon Potter–Elvehjem homogenizer in approx. 10 vol. of 0.32 M sucrose, 5 mM Hepes buffer, pH 7.2, in the presence of protease inhibitors (0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A and 0.05 mM benzamidine) and centrifuged at 500 g for 10 min. The pellet was rehomogenized in

Abbreviation used: IICR, InsP_3 -induced Ca^{2+} release.

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~ 5 vol. of the same buffer and centrifuged as above. The two supernatants were pooled and centrifuged for 20 min at 10000 *g* to remove the mitochondria. The resulting supernatant was again centrifuged for 1 h at 100 000 *g* to give the microsomal pellet, which was resuspended in Hepes/sucrose buffer, snap-frozen in liquid nitrogen and stored at -70 °C until required.

Ca²⁺-ATPase activity was measured by a phosphate liberation assay as described in [14]. [³H]InsP₃ binding studies as described in [15] were undertaken in the absence and presence of 2% (v/v) alcohol. Briefly, the effect of alcohols on the displacement of [³H]InsP₃ were assessed by suspending approx. 0.5 mg of pig cerebellar microsomes in a medium containing 50 mM Tris/HCl, 100 mM KCl, 1 mM EDTA, pH 8.4, in a total volume of 0.5 ml. Total [³H]InsP₃ binding was measured in the presence of 40 nM InsP₃ (the *K_d* value) and non-specific binding was determined in the presence of 10 μM excess unlabelled InsP₃. The samples were incubated for 10 min on ice and centrifuged for 15 min at 15000 *g*, then the pellets were thoroughly washed with distilled water, dried and solubilized in 0.5 ml of Solvable (du Pont) and incubated for 1 h at 37 °C. The radioactivity of the samples was finally determined by scintillation spectrometry.

Measurements of calcium uptake and IICR were monitored by following changes in fluorescence of the calcium indicator dye fluo-3 [15,19,20]. This was then related to changes in Ca²⁺ concentration using the following equation:

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

where *K_d* is the dissociation constant for Ca²⁺ binding to fluo-3 (900 nM at 37 °C, pH 7.2, in 100 mM KCl [20]; this value was unaffected by up to 5% (v/v) alcohol), *F* is the fluorescence intensity of the sample and *F_{min}* and *F_{max}* are the fluorescence intensities of the sample in 1 mM EGTA and 2 mM CaCl₂ respectively. Cerebellar microsomes (0.5 mg) were added to a stirred cuvette containing 2 ml of 40 mM Tris-phosphate, 100 mM KCl buffer, pH 7.2, in the presence of 1.25 μM fluo-3, 10 mM phosphocreatine and 10 μg/ml creatine kinase. Calcium uptake was then initiated by the addition of 1.5 mM MgATP, and once sufficient Ca²⁺ accumulation had occurred further uptake was inhibited by the addition of up to 0.5 mM orthovanadate (which inhibited > 90% of the Ca²⁺ pumps [14,21]). This caused some Ca²⁺ release to occur due to passive leakage; a new steady state was then reached where passive leakage was equal to passive influx, such that the monitored Ca²⁺ concentration was constant throughout the remainder of the experiment. During this period InsP₃ was added and the amount of calcium released was expressed as a percentage of that released by the addition of 10 μg/ml ionophore (A23187). In all experiments in which alcohols were present they were added prior to MgATP addition. Ca²⁺ concentrations were allowed to stabilize after orthovanadate was added, prior to measuring IICR. Typically there was a 20 min incubation of the microsomes with alcohols before InsP₃ addition. It must also be noted that since most of the Ca²⁺ loaded into microsomes is bound to Ca²⁺ buffering proteins (such that free Ca²⁺ is in the 10–100 μM concentration range [22]), and since a small proportion (typically 10–40%) of the accumulated Ca²⁺ is released in our cerebellar microsomes by InsP₃, it is unlikely that the Ca²⁺ gradient across the microsomal membrane would be greatly affected during IICR.

Rapid measurements of IICR were carried out by introducing the microsomes (which had been loaded with Ca²⁺ and had their Ca²⁺ pumps inactivated by the addition of orthovanadate) into the large syringe of a stopped-flow spectrofluorimeter (Applied Photophysics; SX-17MV); InsP₃ was added from a small syringe (drive ratio 10:1). The contents of the two syringes were rapidly

mixed together (the total volume after mixing being 300 μl) and the changes in fluorescence (monitored by excitation at 505 nm and detecting emission above 515 nm using a cut-off filter) were recorded. The fluorescence data were initially adjusted by comparing the changes on the stopped-flow apparatus with identical experiments undertaken on a conventional fluorimeter, such that these traces could be related to fractional Ca²⁺ release. The data were then analysed by non-linear least-squares fitting to a monoexponential equation (software package supplied by Applied Photophysics):

$$f = A \times (1 - \exp^{-kt})$$

where *A* is the maximum amplitude of Ca²⁺ release (fractional Ca²⁺ release) for that particular InsP₃ concentration, *t* is time and *k* is the rate constant for Ca²⁺ release. Over the Ca²⁺ concentration range for which IICR was monitored, the fluorescence changes when related to Ca²⁺ concentration were around the *K_d* for Ca²⁺

Table 1 Effects of alcohols on ATP-dependent Ca²⁺ uptake and Ca²⁺-ATPase activity

The values represent the means ± S.D. of three or more determinations.

Alcohol (% v/v), (mM)	Ca ²⁺ uptake (nmol of Ca ²⁺ /min per mg)	Ca ²⁺ -ATPase activity (nmol of P _i /min per mg)
Control	2.5 ± 0.3	8.9 ± 0.6
Ethanol (1%, 172)	4.6 ± 0.2	9.0 ± 1.0
Ethanol (2%, 343)	5.6 ± 0.3	9.4 ± 0.5
Ethanol (5%, 858)	8.1 ± 0.4	9.6 ± 0.3
Methanol (2%, 494)	4.1 ± 0.2	9.3 ± 0.7
Propanol (2%, 266)	2.8 ± 0.4	6.9 ± 0.5
Propan-2-ol (2%, 260)	3.3 ± 0.3	11.9 ± 0.8
Butanol (0.1%, 11)	2.9 ± 0.4	8.3 ± 0.4
Butanol (2%, 220)	0.6 ± 0.3	6.9 ± 0.3

Figure 1 Effects of ethanol on IICR from pig cerebellar microsomes

Ethanol was incubated with the microsomes in 40 mM Tris-phosphate, 100 mM KCl buffer, pH 7.2, at 37 °C, prior to ATP addition. Ca²⁺ uptake was inhibited by orthovanadate once sufficient Ca²⁺ accumulation had taken place, and InsP₃ at either 0.5 μM (●) or 20 μM (○) was added. The amount of Ca²⁺ release was expressed as a percentage of that released by 10 μg/ml A23187. The data are presented as the means ± S.D. of between three and six determinations.

binding to fluo-3. Over this range fluorescence is linearly related to Ca²⁺ concentration (linear regression coefficient, $r > 0.99$).

RESULTS

Table 1 shows that the rate of Ca²⁺ loading into microsomes was found to vary in the presence of ethanol, although Ca²⁺-ATPase activity was little affected. Methanol and propanol also increased Ca²⁺ uptake to a lesser extent, whereas butanol above 0.1 % (v/v) inhibited Ca²⁺ uptake. It was therefore important that in all experiments we allowed Ca²⁺ to accumulate in the microsomes to similar levels. This was achieved by following Ca²⁺ uptake in the fluorimeter and inhibiting the pumps with up to 0.5 mM orthovanadate once similar levels of Ca²⁺ accumulation had taken place. Upon inhibition with orthovanadate some Ca²⁺ leakage

was observed; this, however, soon reached an equilibrium where leakage and uptake were balanced and no further net flux was observed. Only once this equilibrium was attained was InsP₃ added.

Maximum IICR from pig cerebellar microsomes occurs at between 10 and 20 μ M InsP₃, giving 27 % Ca²⁺ release when compared with that released by the ionophore A23187. Figure 1 shows the effects of different concentrations of ethanol on IICR from pig cerebellar microsomes following the addition of maximal (20 μ M) and submaximal (0.5 μ M) InsP₃ concentrations. The results show that ethanol was equally effective at inhibiting IICR at both InsP₃ concentrations used [half-maximal inhibition at ~ 3.5 % (v/v) ethanol, which corresponds to a concentration of 600 mM].

Figure 2 shows the effects of 2 % (v/v) (343 mM) and 5 % (v/v) (858 mM) ethanol on the percentage IICR with various InsP₃ concentrations. This preparation of pig cerebellar microsomes had an IC₅₀ of 0.50 ± 0.05 μ M and a Hill coefficient of 1.31 ± 0.13 . In the presence of 2 % and 5 % ethanol the IC₅₀ values for IICR were not affected (0.54 and 0.48 μ M respectively) and the Hill coefficients were also little affected (1.36 ± 0.18 and 1.32 ± 0.08 respectively). The only effect on quantal Ca²⁺ release therefore appeared to be on the extent of Ca²⁺ release (i.e. the percentage of Ca²⁺ released). At 20 μ M InsP₃ the Ca²⁺ release decreased from 27.4 % in the absence of ethanol to 16.1 % and 13.2 % in the presence of 2 % and 5 % ethanol respectively.

Table 2 shows the effect of other alcohols on IICR at both constant % (v/v) and molarity. The results show a correlation between the increase in the chain length of the alcohol and the decrease in IICR, whether the alcohols were measured at constant % (v/v) or constant molarity. Butanol caused a much greater inhibition of IICR than other alcohols even at concentrations 20-fold less than ethanol (0.1 %, v/v). Propan-2-ol, however, was less effective than n-propanol, indicating that the potency of these alcohols may also depend on their chemical configuration. [³H]InsP₃ binding studies showed the alcohols to have little effect on ligand binding: binding in the absence of alcohol was 3.4 ± 0.2 pmol/mg while in the presence of 2 % (v/v) alcohols binding was: methanol, 3.2 ± 0.2 ; ethanol, 3.2 ± 0.2 ; propanol, 3.3 ± 0.3 ; propan-2-ol, 3.6 ± 0.4 ; butanol, 2.9 ± 0.5 pmol/mg.

The possibility that these alcohols are affecting Ca²⁺ efflux events was further investigated by analysing the rates at which Ca²⁺ is released upon the addition of InsP₃, using stopped-flow spectrofluorimetry. Figure 3 shows the time course of Ca²⁺

Figure 2 Effects of ethanol on quantal IICR

The effects of InsP₃ concentration on Ca²⁺ release are shown in the absence (□) or presence of 2 % (v/v) (343 mM) ethanol (△) and 5 % (v/v) (858 mM) ethanol (▼). The data are presented as the means $\pm$ S.D. of 3–6 determinations and are expressed as a percentage of the Ca²⁺ released by A23187 (10 μ g/ml).

Table 2 Effects of alcohols on IICR

Ca²⁺ release is expressed as a percentage of that released by A23187. The values represent the means $\pm$ S.D. of at least three determinations. Data in part (b) were obtained using a different microsomal preparation for which maximum Ca²⁺ release was 11.5 % of that induced by A23187.

(a) By % (v/v) of alcohol		Ca ²⁺ release (%)				
InsP ₃ concn. (μ M)	Control	Methanol (2%, v/v)	Ethanol (2%, v/v)	Propanol (2%, v/v)	Propan-2-ol (2%, v/v)	Butanol (0.1%, v/v)
20	27.4 $\pm$ 1.3	21.9 $\pm$ 0.6	16.1 $\pm$ 1.2	13.5 $\pm$ 0.7	17.0 $\pm$ 0.7	7.6 $\pm$ 0.1
0.5	14.5 $\pm$ 0.9	14.7 $\pm$ 0.1	8.2 $\pm$ 0.5	7.3 $\pm$ 0.2	9.4 $\pm$ 0.3	4.4 $\pm$ 0.1
(b) By molarity of alcohol		Ca ²⁺ release (%)				
InsP ₃ concn. (μ M)	Control	Methanol (343 mM)	Ethanol (343 mM)	Propanol (343 mM)		
20	11.5 $\pm$ 0.3	8.4 $\pm$ 0.3	7.6 $\pm$ 0.2	6.8 $\pm$ 0.1

Figure 3 Time-resolved IICR

Experiments were undertaken using a stopped-flow spectrofluorimeter as described in the Materials and methods section. IICR was measured at 20 μM (A) and 0.25 μM (B) InsP_3 (in this preparation of cerebellar microsomes 0.25 μM InsP_3 gave approximately half-maximal release). The figures show the data points and fits using the monoexponential equation when determined in the absence (trace a) or presence of 2% (v/v) (343 mM) ethanol (trace b) and 5% (v/v) (858 mM) ethanol (trace c). Both the rate constants and amplitudes which fit the IICR are given in Table 3. Each trace represents the mean of at least 10 sequential acquisitions.

release at 20 μM (Figure 3A) and 0.25 μM (Figure 3B) InsP_3 . At maximal InsP_3 concentrations Ca^{2+} release reached completion within 2–3 s, which is substantially slower than previous reports using permeabilized rat basophilic leukaemia cells or permeabilized rat hepatocytes [23–25]. However, in the studies with permeabilized rat hepatocytes the Ca^{2+} release data were analysed as being biphasic (biexponential), with fast and slow components [25]. For the data presented here, the rates of Ca^{2+} release could not be conclusively shown to be biphasic, since our data could also be fitted simply to a monoexponential process under all conditions studied. (Statistical analysis of the Ca^{2+} release data presented in Figure 3 showed that all the curves could be fitted to a monoexponential equation with χ^2 values between 0.002 and 0.026 and variance values between 9×10^{-6} and 1.7×10^{-4} . Fitting the data to a biexponential equation showed no improvement, as the χ^2 values in this case were between 0.003 and 0.028 and the variance values were between 1.12×10^{-5} and 1.73×10^{-4} .)

Table 3 Effects of ethanol on the kinetic parameters of IICR

The observed rate constants for Ca^{2+} release were measured by fitting the time courses (see Figures 3A and 3B) to the monoexponential equation given in the Materials and methods section. The amplitudes relate to the fractional IICR caused by the addition of either 0.25 or 20 μM InsP_3 , where 1.00 represents the extent or amount of Ca^{2+} release induced by 20 μM InsP_3 .

	0.25 μM InsP_3		20 μM InsP_3	
	k (s^{-1})	Amplitude	k (s^{-1})	Amplitude
Control	0.38 ± 0.03	0.45	0.93 ± 0.10	1.00
Ethanol (2%, v/v; 343 mM)	0.31 ± 0.06	0.23	1.02 ± 0.10	0.69
Ethanol (5%, v/v; 858 mM)	0.33 ± 0.08	0.15	0.99 ± 0.07	0.46

Although we cannot exclude biexponential Ca^{2+} release for this preparation of microsomes, it was decided that for the sake of simplicity all data should be analysed assuming a monoexponential process. Table 3 summarizes the effects of 2% and 5% ethanol on the rates and amplitudes of IICR at 20 μM and 0.25 μM InsP_3 . The results show that, although the rate of IICR is dependent upon InsP_3 concentration, ethanol exerts a negligible effect on the rate constants but decreases the amplitude of Ca^{2+} release.

DISCUSSION

Ethanol at 5% (v/v) stimulated ATP-dependent Ca^{2+} uptake approximately 3-fold, without dramatically affecting Ca^{2+} -ATPase activity, while the longer-chain butanol at 2% (v/v) inhibited both Ca^{2+} uptake and Ca^{2+} -ATPase activity. The net increase in Ca^{2+} uptake is therefore due to either a decrease in membrane leakage of Ca^{2+} through the phospholipid component of the microsomal membrane or the closing of an unidentified protein pore.

In this study we report that aliphatic alcohols inhibit IICR from pig cerebellar microsomes by decreasing the amount of releasable Ca^{2+} . A correlation also exists between the alcohol chain length and the degree of inhibition of IICR, since butanol exhibits a much greater inhibition compared with methanol, ethanol and propanol. It is unlikely that the effects of these alcohols on IICR are due to changes in osmotic strength experienced by the microsomes upon exposure to relatively high concentrations of alcohols. As alcohols are small, relatively non-polar, molecules they are able to permeate across the microsomal membrane, dissipating any osmotic gradient. In addition, we have shown that other membrane-permeable molecules such as mannitol and glycerol, when used at the same osmotic strength as 5% (v/v) ethanol, had little effect on IICR. However, if membrane-impermeable sucrose was used at this same osmotic strength the microsomes lost their ability to accumulate Ca^{2+} in the presence of ATP, presumably due to vesicle shrinkage and disruption.

The recent findings of Ilyin and Parker [18] showed that ~3% ethanol had a dual effect on IICR from *Xenopus* oocytes, as assessed both directly with fluo-3-injected oocytes and indirectly by monitoring the Cl^- currents evoked by activation of the InsP_3 -mediated Ca^{2+} -sensitive chloride channels. At low concentrations of InsP_3 ethanol was shown to potentiate the release of Ca^{2+} , whereas at higher InsP_3 concentrations ethanol was inhibitory. The authors also showed that methanol had negligible effects on IICR. Longer-chain alcohols such as butanol and hexan-1-ol, however, were shown to strongly inhibit the Cl^- current evoked through Ca^{2+} mobilization at all InsP_3 concentrations used, in agreement with our results. In our measurements

of Ca²⁺ release we did not observe any stimulatory effects of ethanol at any concentration of InsP₃ used, although some stimulation might have occurred with methanol at low InsP₃ concentrations. We have also demonstrated that alcohols are not causing their actions on Ca²⁺ release by affecting InsP₃ binding to its receptor.

The rate of IICR from pig cerebellar microsomes was resolved using stopped-flow techniques and shown to be lower than in earlier reports using permeabilized rat basophilic leukaemia cells and rat hepatocytes [23–25]. This was not due to any effects of the Ca²⁺ pump, since > 90 % of the pumps were inhibited prior to InsP₃ addition. We have calculated the rate constant for Ca²⁺ uptake into microsomes following orthovanadate inhibition to be < 0.01 s⁻¹. As the rate of IICR is much higher (0.3–1.0 s⁻¹) than the rate of Ca²⁺ uptake, especially after orthovanadate inhibition, the rates of IICR would be little affected whether the Ca²⁺ pumps were fully inhibited or 90 % inhibited. In our system at 37 °C, with low InsP₃ concentrations, the release process reached completion after approx. 6 s, whereas with maximal InsP₃ concentrations release was approx. 3-fold faster. With this preparation of pig cerebellar microsomes we found that IICR could best be fitted by assuming a monoexponential process.

Although we have shown that the rate constants for IICR from cerebellar microsomes are consistently lower than those observed in permeabilized rat hepatocytes and rat basophilic leukaemia cells, we have found that they are consistently similar between microsomal preparations. It may seem rather strange to think that a tissue source such as cerebellum, which has probably the highest abundance of InsP₃ receptors compared with other tissues, should have such low rates of release. However, it appears that IICR in intact cerebellar Purkinje neurons begins to occur only at extremely high InsP₃ concentrations (i.e. > 9 μM) [26], which in cerebellar microsomes would induce maximal Ca²⁺ release. These observations must, therefore, indicate that upon disruption of intact cells to produce microsomes some properties of IICR are changed when compared with the process in intact or permeabilized cells.

In some preparations IICR can only be successfully fitted to a biexponential process [27], rather than to a monoexponential process as shown here. As yet we do not fully understand why this should be the case, but it is probably due to subtle differences in preparation procedures, since our microsomal preparations also vary in the amounts of Ca²⁺ which can be released by InsP₃, IC₅₀ values for InsP₃ and the degree of co-operativity. A related phenomenon was recently reported by Hannaert-Merah et al. [28], who showed that the kinetics of InsP₃ binding to and dissociation from cerebellar microsomes were either monophasic or biphasic depending on the preparation used.

From the kinetic data it is clear that the only effect of ethanol is to decrease the amplitude of Ca²⁺ release, and not the rate at which Ca²⁺ is released. If, as has previously been assumed, InsP₃-sensitive Ca²⁺ stores are heterogeneous in their InsP₃ receptors (different isoforms and spliced variants, different phosphorylation states and different sensitivities, etc.), one plausible explanation might be that some stores contain InsP₃ receptors which are more sensitive to ethanol inhibition than others, and therefore are unable to respond to InsP₃ once inhibited at a particular ethanol concentration. This could explain the decrease in the amount of releasable Ca²⁺ with little change in the IC₅₀ value (see Figure 2). It could also explain why ethanol has no effect on the rate constants of Ca²⁺ release. An alternative explanation, which at present cannot be ruled out, is the possibility that the InsP₃-sensitive stores are homogeneous in

their sensitivity to alcohols, but as it is believed that the channel is slowly inhibited upon InsP₃ exposure [29], alcohols may act by speeding up this inhibition event, thus limiting the amount of Ca²⁺ released. However, depending on the rates of channel opening and channel closing (inhibition), this may or may not significantly affect the overall rate constants for Ca²⁺ release as measured here.

Alcohol intoxication may, at least in part, arise from impairment of InsP₃ receptor function, since many neuronal signalling processes require Ca²⁺-mediated events that arise from neurotransmitter stimulation of IICR [13]. In addition alcohols are commonly used as vehicles to dissolve non-polar drugs prior to incubation with cells and subcellular fractions. In the light of our results we advise caution in the use of such solvents when studying Ca²⁺ homeostatic processes.

In conclusion, alcohols are able to inhibit the amount or extent of IICR without any effect on the IC₅₀ for quantal Ca²⁺ release, the rate of Ca²⁺ release or the affinity of InsP₃ binding to its receptor. The inhibition potency increases with alcohol chain length and therefore hydrophobicity, which would suggest that the site of action is either within the hydrophobic core of the receptor or at the lipid-protein interface.

The BBSRC and the British Heart Foundation are acknowledged for financial support. We thank Dr. E. Grimes for her helpful comments.

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