British Journal of Pharmacology (1997) 122, 1661–1668

Differential modulation of rat neuronal nicotinic receptor subtypes by acute application of ethanol

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1 We have studied the effects of acute ethanol (EtOH) exposure on the agonist responses of rat neuronal nicotinic receptors expressed in *Xenopus* oocytes by means of voltage clamp techniques.

2 In some cells, agonist-induced current responses with the $\alpha3\beta4$ subunit combination could be either significantly potentiated or inhibited (range 25% to 237% of control response) by low ethanol concentrations (1–30 mM). At high ethanol concentrations (100–300 mM) robust potentiations were observed (range 135% to 305% of control).

3 The low EtOH concentration effects on the $\alpha3\beta4$ subtype exhibited tolerance with repeated EtOH exposure.

4 In general, the $\alpha3\beta2$, $\alpha4-1\beta2$ and $\alpha4-1\beta4$ subunit combinations were less sensitive to low concentrations of ethanol, but respectively showed potentiations of up to 178%, 226% and 154% at high EtOH concentrations.

5 The $\alpha7$ homomeric receptor was also relatively insensitive at low EtOH concentrations. At high EtOH concentrations, potentiations, inhibitions or no alteration of control agonist response were observed (range 88% to 141% of control).

6 We conclude that all the neuronal nicotinic receptor subunit combinations tested here can be modulated by high concentrations of EtOH in a rapidly reversible manner. This modulation may underlie some of the behavioural effects of ethanol. The $\alpha3\beta4$ subunit combination may be especially sensitive to modulation by low EtOH concentrations. This remarkable sensitivity and plasticity of nicotinic receptors may contribute to a process of mutual reinforcement in nicotine and alcohol addiction.

Keywords: Ethanol; smoking; nicotinic receptors; plasticity; neuronal; ion channels; oocytes; addiction

Introduction

There is a strong correlation between the excessive consumption of both alcohol and nicotine (Kozlowski *et al*., 1993). This correlation is also seen in animal studies of ethanol-nicotine interactions at both behavioural (Dar *et al*., 1993; Blomqvist *et al*., 1996) and genetic levels (De Fiebre & Collins, 1992; Blomqvist *et al*., 1996). Nicotinic receptors are also thought to be involved in ethanol-induced dopamine release in the limbic forebrain (Blomqvist *et al*., 1993). The question therefore arises as to whether there might be direct actions of alcohol on neuronal nicotinic acetylcholine receptor subtypes which underlie the synergism between alcohol and nicotine addiction.

Alcohol is known to potentiate the activity of muscle nicotinic acetylcholine receptors (AChRs) (Arcava *et al*., 1991), and is also able to modulate the activity of a variety of other neuronal receptor subtypes (Samson & Harris, 1992, review; Li *et al*., 1993). Also, acutely applied alcohol has recently been shown to inhibit the chick $\alpha7$ neuronal nicotinic receptor in a non-competitive mechanism involving the amino-terminal domain of the receptor (Yu *et al*., 1996). In other receptors, some aspects of its mechanism of action have been elucidated. For example the ethanol-induced enhancement of the activity of recombinant γ-aminobutyric acid $(GABA)_A$ receptors requires the presence of the $\gamma2L$ subunit (Wafford *et al*., 1991), and is thought to be partly mediated by protein kinase C (Wafford & Whiting, 1992; Lin *et al*., 1994). This effect may also occur in hippocampal neurones in the brain (Weiner *et al*., 1994). Protein kinase C also mediates one of the pathways through which alcohol inhibits the kainate responses of glutamate receptors (Dildy-Mayfield & Harris, 1995), although there is also evidence of an additional adenosine 3′:5′-cyclic monophosphate (cyclic AMP) independent mechanism. However, despite years of intensive effort, there is still considerable debate about how alcohol exerts its effects on receptors. For example, perturbation of the fluidity of annular lipids or a discrete hydrophobic pocket within the receptor are also mechanisms that have been proposed (Franks & Lieb, 1994, review). Ethanol can also alter the activity of phospholipase D (Pai *et al*., 1988). It therefore seems probable that alcohol has more than one mechanism through which it exerts its effects.

In order to characterize these effects, we have examined the action of alcohol on the agonist responses of several neuronal nicotinic AChR subtypes functionally expressed in *Xenopus* oocytes. Some of this work has been published previously in abstract form (Covernton & Connolly, 1995; 1997; Covernton *et al*., 1995).

Methods

Functional expression in Xenopus *oocytes*

Defolliculated oocytes were prepared for injection as described previously (Boulter *et al*., 1987). Diguanosine-triphosphate capped RNA was transcribed *in vitro* from the corresponding DNA template. Up to 20 ng of RNA or 3–5 ng of DNA encoding subunits of nicotinic AChRs were injected into the oocytes in a ratio of approximately 1:1.5 ($\alpha:\beta$). Injection was into the oocyte cytoplasm (RNA) or nucleus (cDNA). Rat subunit cDNAs under the control of the SV40 early promoter in the Flip vector (Bertrand *et al*., 1992) were kindly provided by Dr Robert Duvoisin (Dyson Vision Research Institute, Cornell University, New York). The other subunits were provided by Prof S. Heinemann, Dr J. Boulter and Dr D. Johnson (Salk Institute, U.S.A.). The pGEM HE vector (Liman *et al*., 1992), into which the rat $\alpha7$ subunit was subcloned, was originally provided by Dr Emily Liman (Harvard, U.S.A.). The subunit combinations $\alpha3\beta2$, $\alpha3\beta4$, $\alpha4-1\beta2$, $\alpha4-1\beta4$ and $\alpha7$ were investigated.

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Electrophysiological recording

Two electrode voltage clamp recordings ($V_H = 60$ mV) were obtained by means of an Axon Geneclamp 500, and nicotinic agonist-induced current responses recorded in the presence and absence of a variety of concentrations of ethanol (Spectrosol, Sigma). Responses were recorded directly onto a chart recorder (Gould EasyGraph). Electrodes were pulled from borosilicate glass (GC150-TF, Clark Electromedical) by a Flaming Brown micropipette puller (Sutter Instrument Co.), and broken back to the required resistance ($R_I = 0.5-1$ MΩ, $R_V = 2-5$ MΩ). Pipette solutions had the following composition: current pipette, CsF 0.25 M, CsCl 0.25 M, EGTA 100 mM, pH 7.2; voltage pipette, 3 M KCl. All external recording solutions, both control and those to which drugs had been added, contained in (mM) NaCl 115, $BaCl_2$ 1.8, KCl 2.5, HEPES 10 (pH 7.2) and atropine 1 μM to block endogenous muscarinic currents. Ba^{2+} replaced Ca^{2+} in the external medium in order to minimize the endogenous Ca^{2+}-activated Cl^- conductance, which manifests itself as a transient net inward current at $V_H = 60$ mV. This substitution was made in case a possible effect of ethanol on the Ca^{2+}-activated Cl^- conductance might be misinterpreted as a direct effect on nicotinic AChRs. A gravity-driven manual switching system allowed either drug or control solution to be rapidly perfused (25 ml min^{-1}) through an effective bath of volume 0.3 ml. All reagents were obtained from Sigma-Aldrich Chemical Company.

Drug application protocol and data analysis for EtOH modulation

Initially, reproducible current responses were obtained to the standard agonist concentration. All drug applications were made at 5 min intervals. For all the subtypes tested, agonist responses in Ba^{2+} often exhibited a gradual 'run-up' or 'run-down' depending on the oocyte. Oocytes which could not reach a reproducible control response (i.e. within 10% of the previous response) were not used for analysis. After a reproducible control agonist response was obtained, the same concentration of agonist was co-applied with alcohol. Following this a recovery control response was obtained. Data were not used from the cells where the immediate recovery response was not within 20% of the previous control value.

Agonist concentrations routinely used were in the range 1–30 μM. If very high concentrations of agonist were used with short application intervals, the test and recovery responses could be badly affected by desensitization. If the interval between applications was made long enough for recovery from high agonist concentrations, then run down of the response could also jeopardize estimation of the degree of modulation (data not shown). Therefore, in order to obtain the most reliable estimation of the degree of modulation of the agonist response by ethanol, we used low μM concentrations of agonist combined with short time intervals between drug applications.

As discussed below, there was evidence of the development of tolerance of the agonist response to modulation by ethanol. Therefore, only the 'first-exposure' co-applications of agonist and ethanol were used to construct concentration-response relationships.

Results

Subtype comparison of the effects of ethanol

As mentioned above, due to problems of desensitization and run-down, it was decided to make comparisons at 1–10 μM ACh for the heteromeric subtypes. This is approximately equal to a level of $\leqslant EC_{25}$ for all the subtypes if their concentration-response curves are fit with a single component (data not shown), and is low enough to allow adequate recovery from desensitization between agonist applications. For the homomeric α7 receptor, nicotine (10–30 μM) was used in preference to ACh due to the relatively low potency of the latter agonist. This was also equivalent to a value of $\leqslant EC_{25}$. Some results from early experiments on the α3β4 combination with 10 μM nicotine have also been included in the ethanol concentration–% control plots in Figure 6a, but these have been identified by open symbols.

Modulatory actions of ethanol at low concentrations on responses of the α3β4 subunit combination

For the α3β4 combination, low concentrations of ethanol (1–30 mM) were found to have different effects in different oocytes. In some cases, ethanol potentiated the response of the α3β4 combination to nicotinic agonists. In some others, the agonist response was inhibited by ethanol. Figure 1 shows examples of inward current responses from 4 different oocytes revealing both potentiation and inhibition of α3β4 responses at remarkably low concentrations of EtOH (1–3 mM). In the top trace 1 mM EtOH potentiated the response to 3 μM ACh to 136% of the control value. In the second trace 1 mM EtOH inhibited the response to 10 μM ACh to 85% of control. In the third trace 3 mM EtOH potentiated the response to 3 μM ACh to 148% of control. In the bottom trace 3 mM EtOH inhibited the response to 1 μM ACh to 68% of control. Despite the problem of tolerance (see Figure 5), these effects were qualitatively reproducible within a given oocyte. For example, in one oocyte, three consecutive applications of 1 mM ethanol + 10 μM acetylcholine were made. The average (±s.e.) of the three responses in the presence of ethanol was 148 ± 2.08 nA. This was significantly greater ($P < 0.005$, Student's t test) than the average of the control current responses (103.3 ± 1.5 nA). When the same concen-

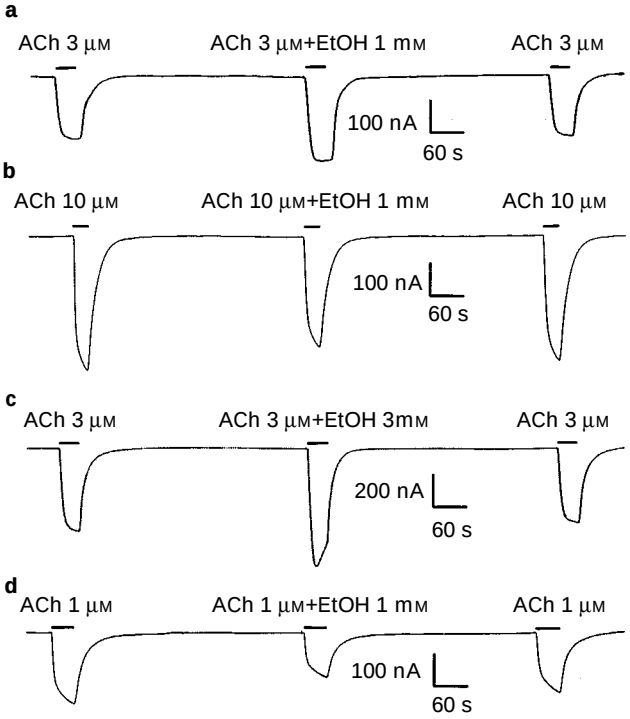

Figure 1 Low concentrations of ethanol both potentiated and inhibited the response of the α3β4 combination to nicotinic agonists. Inward current responses from 4 separate oocytes showing both potentiation and inhibition of α3β4 responses at low concentrations of EtOH (1–3 mM) are presented. In (a), 1 mM EtOH potentiated the response to 3 μM ACh to 136% of the control value. In (b), 1 mM EtOH inhibited the response to 10 μM ACh to 85% of control. In (c), 3 mM EtOH potentiated the response to 3 μM ACh to 148% of control. In (d), 3 mM EtOH inhibited the response to 1 μM ACh to 68% of control. No discernible currents were observed when EtOH was applied alone at concentrations of less than 100 mM.

trations of ethanol and acetylcholine were repeatedly applied to a different oocyte, consistent inhibitions were seen. In this case, the 3 responses in ethanol averaged only 346 ± 2.6 nA, significantly lower ($P < 0.05$) than the control response of 397.5 ± 10.8 nA.

The fact that strong modulatory effects were only observed in some oocytes could cause them to be overlooked. With 1 mM EtOH, 3 out of 8 oocytes showed effects which exceeded a 15% potentiation or inhibition of the control response, and for 3 mM EtOH there were only 2 out of 9 oocytes. Therefore, to obtain an average of the agonist responses in the presence of ethanol from all the oocytes tested, the data from potentiated, inhibited and unaffected cells were combined together. Unsurprisingly, this population mean was not significantly different from the average control result. However, as shown above, this population result concealed the fact that agonist responses within individual oocytes could be significantly altered by low concentrations of ethanol. The mean inward current ($\pm$s.e.mean) obtained for $\alpha 3\beta 4$ with 3 μM ACh was 388.3 ± 40.8 nA ($n = 16$), and no discernible currents were observed when EtOH was applied alone at concentrations equal to or less than 30 mM ($n = 22$).

Figure 2 shows inward current responses from 2 different oocytes revealing the extremes of potentiation and inhibition of $\alpha 3\beta 4$ responses that were sometimes observed at the same ethanol concentration (in this case 30 mM ethanol). In the top trace 30 mM EtOH potentiated the response to 1 μM ACh to 237% of the control value. In the bottom trace 30 mM EtOH inhibited the response to 10 μM nicotine to 25% of control. Thus, both nicotine and ACh can modulate the activity of the $\alpha 3\beta 4$ combination. Therefore, the effects we observed are independent of the agonist used.

Effects of high ethanol concentrations on $\alpha 3\beta 4$

At the upper end of the range (100 – 300 mM EtOH), robust potentiation of the agonist-induced current was observed on every occasion. An example of this is shown in Figure 3, where 300 mM EtOH potentiated the response to 1 μM ACh to 305% of the control value. At ethanol concentrations less than 100 mM, ethanol alone had no effect on membrane current in the absence of agonist. However in Figure 3, 300 mM EtOH alone caused a very small outward current. Such currents were often observed at EtOH concentrations of 100 and 300 mM, and were also seen in uninjected oocytes. The mean first exposure value in all oocytes of current produced by 300 mM ethanol alone was 5.4 ± 0.9 nA ($n = 7$). This outward current would tend to diminish fractionally the measured inward current generated by application of agonist plus ethanol to the oocyte. Since the largest current ever observed for alcohol alone was less than 3.5% of the smallest current response to agonist + alcohol for $\alpha 3\beta 4$, the agonist + alcohol values were not adjusted for a possible contribution to the current by ethanol alone. Similarly, the agonist + alcohol responses of the other combinations were not adjusted. Figure 3 also shows the effect of a repeated co-application with 300 mM EtOH on the same oocyte exhibiting a similar level of potentiation.

Both potentiation and inhibition can occur in the same cell

Figure 4 shows an example of $\alpha 3\beta 4$ receptor agonist-induced responses exhibiting both inhibition and potentiation by different concentrations of EtOH in the same oocyte. The traces run sequentially from top to bottom. At 3 mM EtOH the response to 1 μM ACh was inhibited to 68% of the control value (first exposure to EtOH). At 30 mM EtOH the response to 1 μM ACh was inhibited to 67% of the control value. However, at 300 mM EtOH the response to 1 μM ACh was potentiated to 172% of the control value.

Development of tolerance

Tolerance was defined as a decrease in the degree of ethanol modulation of an unchanging control response following repeated applications of ethanol. An example of this is shown in Figure 5. The first two $\alpha 3\beta 4$ responses are the first and second exposure of the oocyte to agonist + ethanol (1 mM in this case) and are potentiated to a similar level (145% of control). The third trace shows the effect of a 1 mM EtOH co-application later on in the same recording after several higher EtOH concentrations (up to 100 mM) had been applied. The potentiating effect of the 1 mM EtOH had been abolished. This tolerance was not observed in every cell, but it invalidated a protocol of carrying out agonist concentration-response curves in the presence of ethanol. Nor was it valid at a given agonist

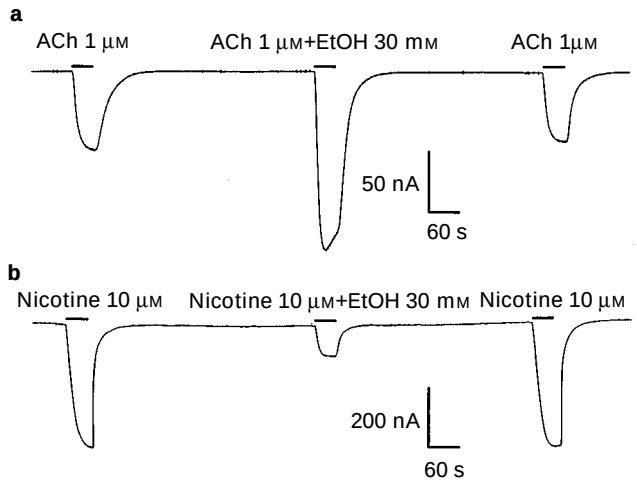

Figure 2 In different oocytes expressing the $\alpha 3\beta 4$ combination, a large potentiation or inhibition was observed at the same ethanol concentration. In (a), 30 mM EtOH potentiated the response to 1 μM ACh to 237% of the control value. In (b), in a different oocyte, 30 mM EtOH inhibited the response to 10 μM nicotine to 25% of control. These effects were not dependent on the type of nicotinic agonist used.

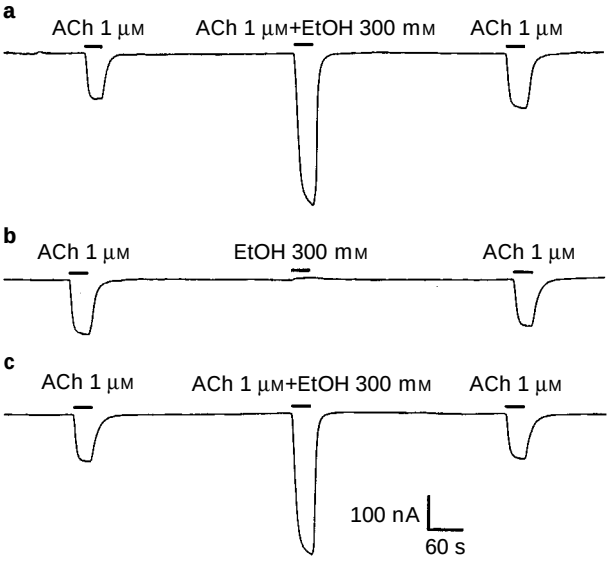

Figure 3 A continuous trace showing the reproducibility of robust potentiations produced by the co-application of concentrations of alcohol to the $\alpha 3\beta 4$ combination. In (a), the response to acetylcholine was potentiated to 305% of the control value by 300 mM ethanol. However, in (b) 300 mM ethanol alone only resulted in a small outward current. (c) Shows a repeated co-application on this cell. This again resulted in potentiation to 305%.

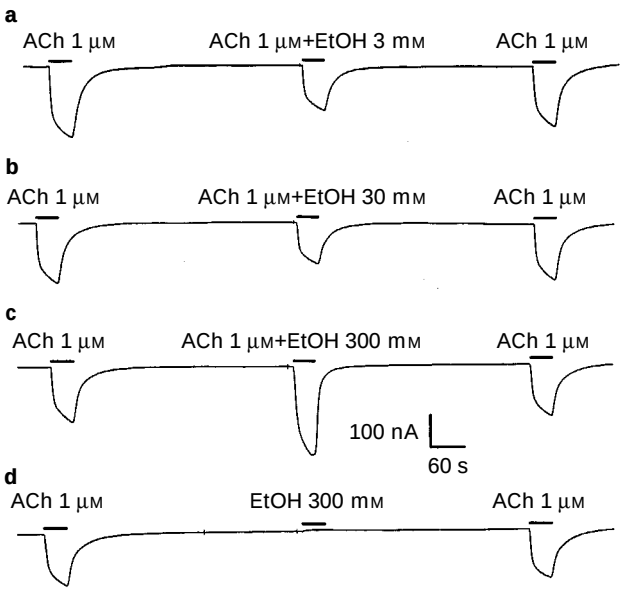

Figure 4 Example of $\alpha 3\beta 4$ receptor agonist-induced responses exhibiting both inhibition and potentiation by different concentrations of EtOH in the same oocyte. The traces run sequentially from top to bottom. At 3 mM EtOH the response to 1 μM ACh was inhibited to 68% of the control value. At 30 mM EtOH the response to 1 μM ACh was inhibited to 67% of the control value. However, at 300 mM EtOH the response to 1 μM ACh was potentiated to 172% of the control value.

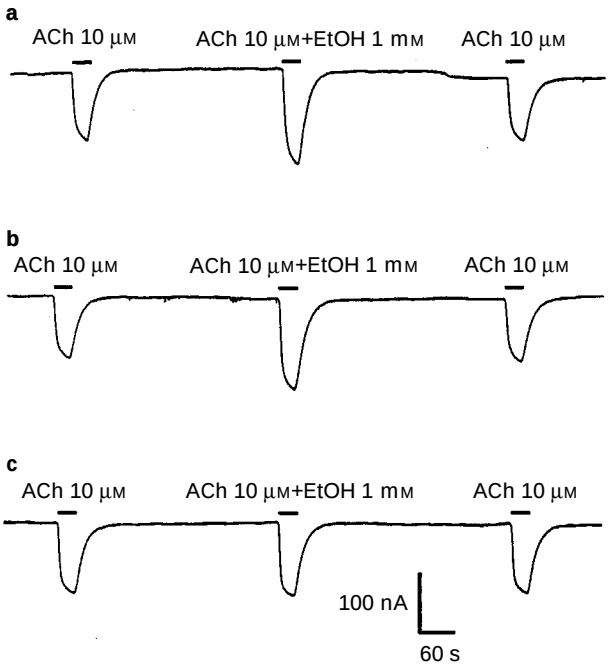

Figure 5 Repeated exposure to low concentrations of ethanol led to the development of tolerance in some cells. In this example the $\alpha 3\beta 4$ responses are all from the same oocyte and run sequentially from top to bottom. In (a), the first co-application of 10 μM ACh and 1 mM EtOH to this cell revealed a potentiation to 145% of the control agonist response. This was followed by a second co-application, (b) in which approximately the same level of potentiation was seen. Following these two co-applications, several higher concentrations of EtOH (up to 100 mM) were applied. Although the magnitude of the control response remained stable throughout, (c) shows that after these high EtOH concentrations the potentiating effect of 1 mM EtOH was no longer evident. Thus tolerance to the potentiating effect of ethanol had occurred.

concentration, to compare effects seen with the first exposure to ethanol with subsequent effects in individual oocytes. To overcome these problems, we adopted the 'first exposure' approach (see Methods) when seeking to compare the actions of ethanol on different receptor subtypes.

Effects of entire clinically relevant range of ethanol concentrations on $\alpha 3\beta 4$ responses

Figure 6a shows a scatter plot of all the 'first exposure' responses of $\alpha 3\beta 4$ receptors in the presence of EtOH. The magnitude of the responses is expressed as a % of the control response. The control response was defined as the average of the responses to agonist alone immediately before and after the first agonist co-application with ethanol. Superimposed on this scatter plot are the points representing the means of the responses in the presence of a particular concentration of ethanol. Each data point represents a single oocyte, and each concentration represents responses from at least 3 different batches of oocytes. Note the large scatter of data points at the low EtOH concentrations (<100 mM) reflecting the reproducible potentiations and inhibitions seen in some oocytes as well as the less affected responses found in others. The overall mean is therefore close to 100%. The variability of modulation by low concentrations of ethanol was not dependent on the batch of oocytes or preparation of RNA from which the responses were obtained, but seemed to be associated with individual oocyte variation within a batch. However, at EtOH concentrations of 100 mM and above only large potentiations were observed. At 100 mM EtOH the mean averaged response to ACh was increased to $146.2 \pm 4.15\%$ (range $= 135.7 - 161.0\%$) of the control value ($n = 5$, $P < 0.0005$), and at 300 mM EtOH the mean averaged response to ACh was increased to $249.8 \pm 16.5\%$ (range $= 206.5 - 305.1\%$) of the control value ($n = 5$, $P < 0.001$).

Actions of ethanol on $\alpha 4 - 1\beta 4$ receptors

The mean inward current obtained for $\alpha 4 - 1\beta 4$ with 1 μM ACh was 1227.1 ± 154.7 nA ($n = 17$). Figure 6d shows the effects of ethanol on $\alpha 4 - 1\beta 4$ responses. It displays a scatter plot with superimposed mean data plot showing the effect of all 'first exposure' agonist-EtOH co-applications relative to control (100%) for the $\alpha 4 - 1\beta 4$ subunit combination. Note also that the average potentiations at EtOH concentrations of 100 mM and above were significantly ($P < 0.05$) lower than those obtained with the $\alpha 3\beta 4$ subunit combination (see Table 1). Each data point represents an individual oocyte (data from a total of 40 oocytes were used for this plot).

Figure 7a shows typical inward current responses obtained from a *Xenopus* oocyte injected with the $\alpha 4 - 1\beta 4$ subunit combination, showing potentiation of the response to 10 μM ACh when co-applied with 300 mM EtOH. The potentiation in this example is only 143% of the control value, which is close to the mean value of $143.3 \pm 2.94\%$ ($n = 6$).

$\alpha 4 - 1\beta 2$ receptors

Figure 6b shows the effects of clinically relevant concentrations of ethanol on $\alpha 4 - 1\beta 2$ responses. The mean inward current obtained for $\alpha 4 - 1\beta 2$ with 10 μM ACh was 349.5 ± 50.0 nA ($n = 27$). Like the combination $\alpha 4 - 1\beta 4$ there appears to be little effect of EtOH at concentrations less than 100 mM, but potentiations were observed at this concentration and above. Agonist-induced current responses for the $\alpha 4 - 1\beta 2$ subtype were increased to $113.3 \pm 3.71\%$ ($P < 0.05$, Student's t test, $n = 5$, range: $108.1 - 127.9\%$) of the control value by 100 mM EtOH, and increased to $188.9 \pm 14.1\%$ ($P < 0.005$, $n = 5$, range: $143.3 - 225.6\%$) by 300 mM EtOH. The potentiation at 300 mM EtOH was significantly lower than that of the $\alpha 3\beta 4$ combination ($P < 0.05$), but also significantly higher than that of the $\alpha 4 - 1\beta 4$ combination ($P < 0.05$). Each data point re-

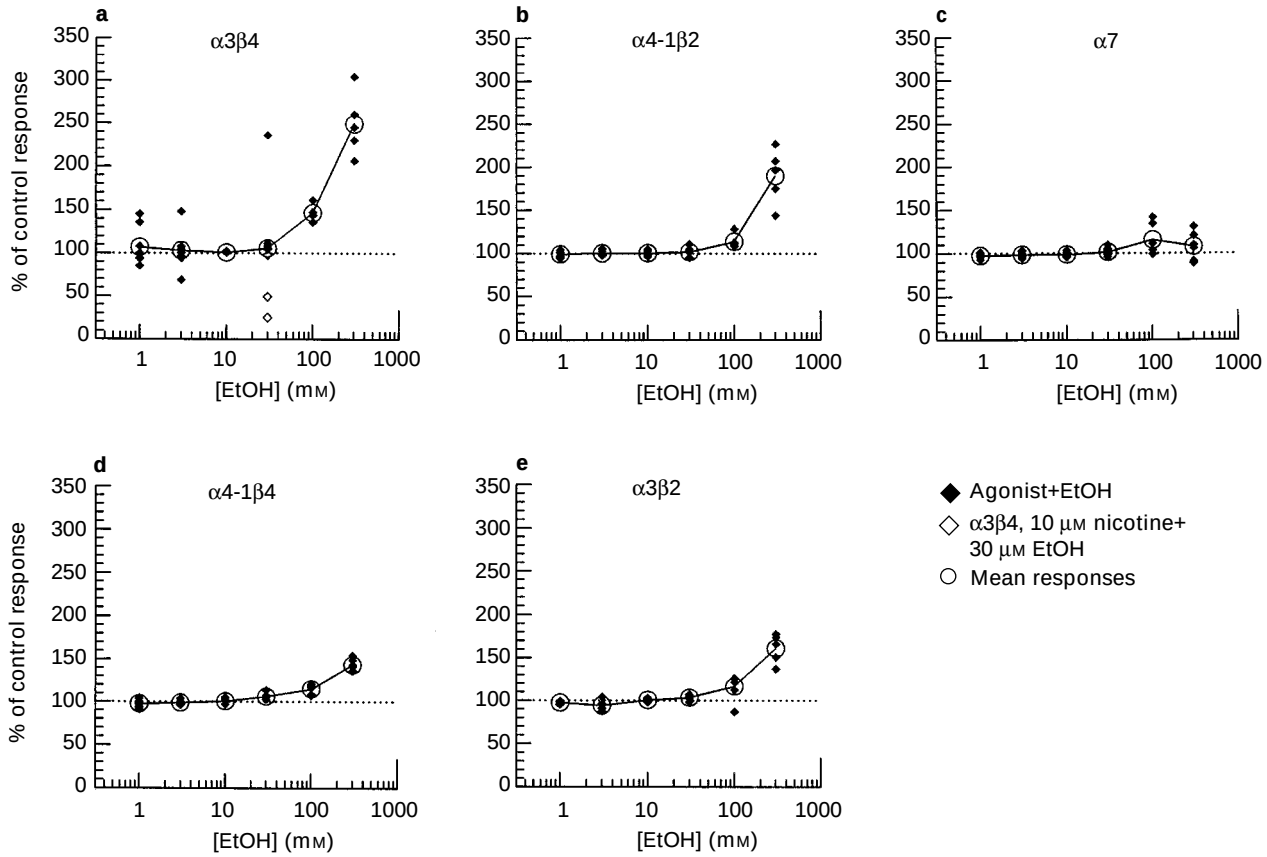

Figure 6 Scatter plots of individual cell responses to 'first shot' agonist-ethanol (EtOH) co-applications. Acetylcholine was the agonist for the paired subunit combinations (except for the indicated points on the $\alpha3\beta4$ plot) and nicotine was the agonist for the homomeric $\alpha7$ receptor. Each data point represents a single oocyte, and each concentration represents responses from at least 3 different batches of oocytes. At each ethanol concentration point, the minimum number of oocytes was 6 for concentrations less than 100 mM, and a minimum of 5 observations for concentrations of 100 mM and 300 mM. Superimposed on this data are symbols indicating the mean response to agonist-ethanol applications at a particular concentration (mean response). The data points are expressed as a % of the control agonist alone responses. The control value (100%) is indicated by a dotted line on the plot. Note that in these experiments, apart from the $\alpha3\beta4$ combination, EtOH had relatively little effect on the agonist responses when applied at concentrations of less than 30 mM. The largest potentiations at EtOH concentrations greater than 30 mM were also seen with the $\alpha3\beta4$ subunit combination.

presents an individual oocyte (a total of 34 oocytes for this plot).

α3β2 receptors

Figure 6e shows the effects of ethanol on $\alpha3\beta2$ responses. The mean inward current obtained for $\alpha3\beta2$ with 10 μM ACh was 91.1 ± 8.7 nA ($n=35$). Like the combination $\alpha3\beta2$ there appeared to be little effect of EtOH at concentrations less than 100 mM, and potentiations were observed at this concentration and above. However, a single inhibition (to 87% of control) was observed at 100 mM EtOH. Agonist-induced current responses for the $\alpha3\beta2$ subtype were increased to $116.2 \pm 6.3\%$ ($P=0.05$, Student's t test, $n=6$, range: 86.7–126.2%) of the control value by 100 mM EtOH, and increased to $161.0 \pm 7.6\%$ ($P<0.002$, $n=5$, range: 136.8–177.6%) by 300 mM EtOH. Each data point represents an individual oocyte (a total of 35 oocytes for this plot).

Ethanol effects on homomeric α7 receptors

Like the $\alpha3\beta2$, $\alpha4-1\beta2$ and $\alpha4-1\beta4$ combinations, the $\alpha7$ homomer was not strongly modulated at low concentrations of ethanol (1–30 mM) under these conditions (Figure 6c). At concentrations above 30 mM the effects were more varied than the heteromeric combinations, with some oocytes exhibiting potentiations, some small inhibitions and others apparently not affected. With 100 mM EtOH the mean response was $115.5 \pm 7.31\%$ ($n=6$, range: 98.9–141.6%), and with 300 mM EtOH the mean response was $107.6 \pm 6.7\%$ ($n=6$, range: 88.4–130.7%). An example of potentiation with 300 mM EtOH is shown in Figure 7b. The mean inward current obtained for $\alpha7$ with 10 μM nicotine was 188.6 ± 22.4 nA ($n=36$).

The effects of high concentrations of ethanol on all the subunit combinations are summarized in Table 1.

Rare effects of ethanol on nicotinic receptors

As discussed above, to avoid problems with tolerance, we adopted a 'first-exposure' protocol. However, during early studies with repeated ethanol exposure some rare effects were observed for the $\alpha3\beta4$ combination. In one case there was a cumulative inhibition from 3–100 mM EtOH. In another, robust potentiations were observed at 3–30 mM EtOH but the extent was inversely proportional to the concentration. In a third example a robust inhibition was observed with 30 mM EtOH, but over a prolonged period, the control response exhibited marked 'run-down' and the EtOH switched to potentiation relative to control. Also, as shown in Figure 6e, we observed a 'first exposure' example of inhibition of $\alpha3\beta2$ at 100 mM ethanol. In a preliminary experiment, $\alpha4\beta4$ responses were inhibited by ethanol in a single oocyte. These rare events suggest that there can be occasional oocytes in which exceptional behaviour occurs.

However, with the more consistent, 'first exposure' Ca^{2+} free conditions employed here, four different phenomena become apparent: (i) variable potentiation at lower ethanol concentrations, (ii) variable inhibition at lower ethanol con-

Table 1 Effects of high concentrations of ethanol (EtOH) on the responses of all the nicotinic subunit combinations to agonist

Subtype	*[EtOH]* (mM)	*Mean*	n	*Range*	*P-value*
$\alpha3\beta4$	100	146.2±4.15	5	135.7, 161.0	<0.0005
$\alpha4$-1$\beta4$	100	114.8±2.36	6	106.7, 120.6	<0.002
$\alpha3\beta2$	100	116.2±6.27	6	86.70, 126.2	0.05
$\alpha4$-1$\beta2$	100	113.3±3.71	5	108.1, 127.9	<0.05
$\alpha7$	100	115.5±7.31	6	98.90, 141.6	NS
$\alpha3\beta4$	300	249.8±16.5	5	206.5, 305.1	<0.0005
$\alpha4$-1$\beta4$	300	143.3±2.94	6	135.7, 154.2	<0.0001
$\alpha3\beta2$	300	161.0±7.62	6	136.8, 177.6	<0.002
$\alpha4$-1$\beta2$	300	188.9±14.1	5	143.3, 225.6	<0.005
$\alpha7$	300	107.6±6.70	6	88.40, 130.7	NS

The mean values±s.e.mean of the agonist response in the presence of 100 and 300 mM ethanol are expressed as a % of the control response in the absence of ethanol. n=the number of oocytes in which first-exposure responses were studied. In nearly all cases robust potentiations were seen which were significantly greater than the control responses (Student's t test of the mean, Mini-Tab).

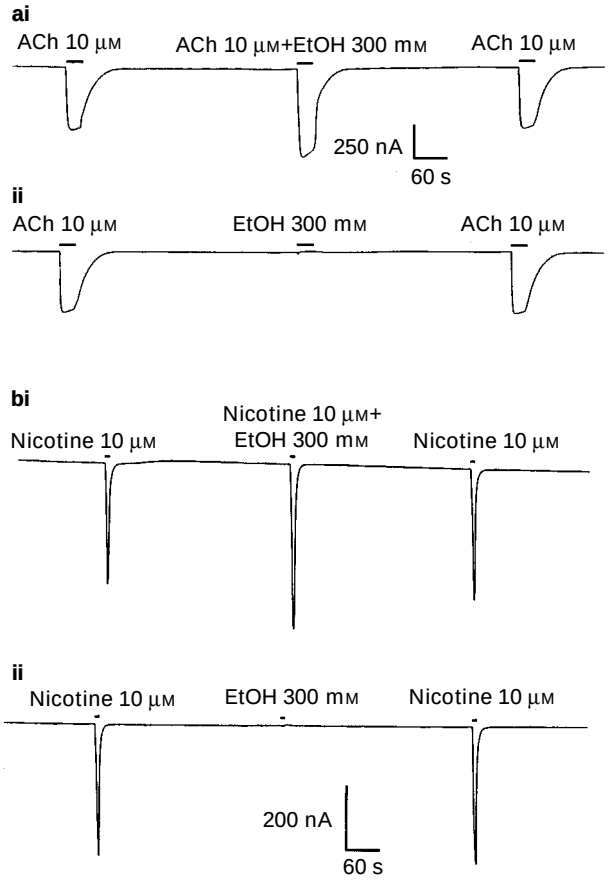

Figure 7 (a,i) Typical inward current responses obtained from a *Xenopus* oocyte injected with the $\alpha4-1\beta4$ subunit combination, showing potentiation of the response to 10 μM ACh when co-applied with 300 mM EtOH. The potentiation in this example was only 142% of the control value, which was close to the mean value of 143.3% (n=6). This was significantly less (P<0.005) than the mean value of 249.8% for the $\alpha3\beta4$ response (n=5). As with all of the effects of ethanol, there was complete and rapid recovery of the control response. In (a,ii), a subsequent application of 300 mM ethanol alone showed only a small outward current. (b) Example of an $\alpha7$ response showing potentiation by 300 mM EtOH to 131% of the control value (i). Again the subsequent effect of 300 mM ethanol alone was barely detectable (ii).

centrations, (iii) the development of tolerance, and (iv) a consistent potentiation at the highest ethanol concentrations.

Discussion

Modulation of neuronal nicotinic AChRs by ethanol

The data presented here suggest that different neuronal nicotinic receptor subtypes can differ in their characteristics of modulation by physiologically encountered concentrations of ethanol. Under the conditions employed in these experiments, the different subtypes appeared to fall into three categories: (i) $\alpha3\beta4$-type effects are characterized by the presence of both potentiation and inhibition at low ethanol concentrations in some cells, and robust potentiation at high ethanol concentrations; (ii) generally $\alpha3\beta2$, $\alpha4-1\beta2$ and $\alpha4-1\beta4$ receptors are less sensitive at low concentrations, but show potentiation at high EtOH concentrations; (iii) $\alpha7$-type receptors are relatively insensitive at low EtOH concentrations, but show both potentiation and inhibition at high EtOH concentrations. However, this characterization may be somewhat arbitrary. The low concentration effects observed with $\alpha3\beta4$ were relatively rare events. Therefore, it is possible that under other conditions, such as in the presence of Ca^{2+} (Dildy-Mayfield & Harris, 1995), the other subtypes may show greater sensitivity than they have in this particular set of experiments.

In contrast to the variability of the effects of low ethanol concentrations, potentiation of responses at high ethanol concentrations was nearly always seen. However, Figure 5 and Table 1 demonstrate that the average magnitude of the potentiation produced by 300 mM ethanol was different for different combinations. The order of decreasing sensitivity to ethanol was $\alpha3\beta4>\alpha4\beta2>\alpha3\beta2>\alpha4\beta4>\alpha7$. The responsiveness of the $\alpha3\beta4$ combination was considerably greater than that of any other. This cannot be attributed to the presence of an independent site on the $\beta4$ subunit, since the $\alpha4\beta4$ combination showed the least tendency to potentiation of any of the heteromeric combinations. Nor can it be attributed solely to the presence of the $\alpha3$ subunit, as the $\alpha3\beta2$ combination showed less potentiation than the $\alpha4\beta2$ combination. It would seem that both $\alpha3$ and $\beta4$ subunits are required. Perhaps both subunits contribute juxtaposed domains which together form a binding site for alcohol. In other combinations, the domains may be less well aligned and the synergism would not occur.

Studies on the responses of nicotinic AChRs in ganglia, which also express $\alpha3$ and $\beta4$ subunits, show that the activity of the receptors can be both upregulated and downregulated (Valenta *et al*., 1993; Gurantz *et al*., 1994). We also see this with $\alpha3\beta4$ receptors expressed from cDNAs in oocytes. The variability of the degree of modulation of $\alpha3\beta4$ responses by low concentrations of ethanol has several interesting implications. If ethanol worked simply by acting on a hydrophobic pocket in the protein or on the annular lipid to disrupt protein structure, then we would perhaps expect more consistency in the degree and direction of current modulation. The inconsistency at low concentrations tends to argue against a simple change in membrane fluidity as the sole mechanism. Also, studies on the *Torpedo* receptor in lipid vesicles suggest that the lipid-protein interface, at least deep in the bilayer, is quite insensitive to the presence of ethanol up to 0.9 M (Abadji *et al*., 1994). One possibility is that the rapid potentiation and inhibition of nicotinic responses at low concentrations of ethanol may be mediated by intracellular signalling pathways. This may involve intimate association of the receptor with a regulatory protein, such as occurs between the N-methyl-D-aspartate (NMDA) receptor and protein tyrosine kinase Src (Yu *et al*., 1997).

The $\alpha3$ and $\beta4$ subunits are not just restricted to ganglia, but are also found throughout the brain (Dinely-Miller & Patrick, 1992). $\alpha3$ and $\beta4$ subunits also occur together in receptors in the mammalian nervous system (Flores *et al*., 1996). In oocytes, this combination is characterized by a long open time

(Papke & Heinemann, 1991) and like all neuronal nicotinic receptors, is more permeable to Ca^{2+} than muscle AChRs (Mulle *et al*., 1992; Vernino *et al*., 1992; 1994). Nicotinic single channel activity with analogous characteristics has been observed in the medial habenula (Mulle *et al*., 1992; Connolly *et al*., 1995), where $\alpha3$ and $\beta4$ subunits are also expressed (Duvoisin *et al*., 1989), and so it seems probable that the observations described here will have relevance to the actions of ethanol on native nicotinic AChRs containng $\alpha3$ and $\beta4$ subunits in brain tissue. Both these subunits are also expressed in PC12 cells (Boulter *et al*., 1990), where Nagata *et al*. (1996) have recently shown that low concentrations of ethanol (30 μM to 10 mM) can produce variable effects of potentiation and inhibition of nicotinic responses.

The effects of ethanol on the rat $\alpha7$ homomeric receptor presented here appear to contradict those obtained for the chick $\alpha7$ in another study. In the work of Yu *et al*. (1996) ethanol caused a dose-dependent inhibition of the nicotine-induced current response ($IC_{50} = 33$ mM), whereas we observed mixed inhibition/potentiation at concentrations above 30 mM. The reasons for this difference are unclear, as the agonist concentrations used were similar (10 μM vs 10–30 μM nicotine) and acute ethanol applications were used (although not using a 'one-shot' protocol). Also, the use of higher agonist concentrations would not seem to explain the differences. In one cell, the response of rat $\alpha7$ to 100 μM nicotine was only reduced by 5% in the presence of 100 mM ethanol. A second response in the same cell to 300 μM nicotine was reduced by only 2.5%. In a second cell the response to 300 μM nicotine was not altered in the presence of 300 mM ethanol (data not shown). It would therefore seem possible that there is a species difference between the rat and chick $\alpha7$ receptors in their pattern of ethanol modulation. Several pharmacological differences between these two receptors have previously been described – for instance the agonist 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) is a potent near-full agonist on the rat $\alpha7$ receptor, but a very weak partial agonist on the chick $\alpha7$ receptor (Bertand *et al*., 1992; Séguéla *et al*., 1993), even though there is considerable (90%) amino acid sequence homology between them (Couturier *et al*., 1990; Séguéla *et al*., 1993). However, DeFiebre *et al*. (1995) suggested that such inhibition by EtOH may also occur with the rat $\alpha7$ receptor.

The suggestion that the observations described here may have importance beyond the oocyte is reinforced by the fact that mecamylamine, a nicotinic antagonist, can antagonize the mesolimbic dopamine-activating properties of ethanol (Blomqvist *et al*., 1993). Therefore, it seems possible that alcohol enhancement of nicotinic receptor activity in the mesolimbic pathway may contribute the mutual reinforcement of drinking and smoking behaviour. Similarly, it is possible that the modulation of neuronal nicotinic receptor subtypes may contribute to the induction of alcohol-dependence due to chronic high alcohol exposure. The concentration of ethanol at which the potentiating effect starts to occur in all the heteromeric receptors tested here (and sometimes with the $\alpha7$ receptor) is equivalent to that regularly experienced by heavy drinkers (i.e. <30 mM, which is equivalent to approx. 10 units; 1 unit = 8 g = 10 ml EtOH).

In rat substantia nigra reticulata and ventral palladium, Criswell *et al*. (1993) examined the nicotine-induced changes in neuronal firing rate. In 7 of 9 cells which were excited by nicotine, the increase in firing rate was enhanced by alcohol. However, Frölich *et al*. (1994) noted that alcohol could inhibit the excitatory effects of nicotine, kainate and NMDA on neuronal firing rates in rat locus coereleus, where $\alpha3\beta4$ is also expressed. From these results it is apparent that the effects of ethanol on nicotinic receptors are not uniform in the brain, but may be subtype specific, or depend upon the intracellular signalling status of the cell under investigation.

Criswell and colleagues also noted that the degree of inhibition of the NMDA response declined with prolonged incubation with ethanol. We have observed similar evidence of an initial strong sensitivity to low concentrations of ethanol, which fades with repeated exposure. This raises the possibility that if an individual rapidly consumes even a small amount of alcohol, then there could be a window of time before tolerance sets in during which the person's performance is strongly affected. For a short period, driving ability could be compromised even though the blood alcohol level is well below the legal limit.

Another implication of these results is that in the brain, the control of the activity of nicotinic AChRs may be highly dynamic and specific for each subunit combination. The diversity of the MIII–MIV cytoplasmic domains may mediate some of this plasticity, enabling each receptor subtype to respond rapidly to a particular set of intracellular signalling influences. Unfortunately, dialysis of neurones during patch clamp may remove or disrupt some of these signalling pathways.

In the light of the present results, it is tempting to suggest that the direct effects of alcohol on neuronal nicotinic receptors is responsible for the intense need to smoke that some people feel when drinking alcohol. It will be informative to determine whether it is the inhibitory effect or the potentiating effect which is important, and again which subtypes are involved. The answers to these questions will help define the mechanisms of reward in drug dependence. They may also help in the design of more effective strategies for smoking cessation, for if the effects described here occur in the brain, then there may be a case for advising those hoping to give up smoking that they should avoid heavy drinking at all costs!

We would like to acknowledge the support of The Wellcome Trust, The M.R.C. (U.K.), The Royal Society, S.H.E.R.T., The Strathclyde Molecular Biology Laboratory, The Strathclyde University Research and Development Fund, and the technical contributions of Fiona Kempsill and Angela Garman. We would like to thank Dr Diane Dixon for supportive work in the preliminary stages of this study, and Prof Steve Heinemann, Drs Jim Boulter & David Johnson (Salk Institute, U.S.A.), and Dr Robert Duvoisin (Dyson Vision Research Institute, Cornell University, New York) for the provision of AChR subunit cDNAs. We also thank Prof David Colquhoun and Dr Alasdair Gibb (University College London, U.K.) for helpful advice and discussions and for the generous provision of analytical software (DC).

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(Received March 11, 1997
Revised July 29, 1997
Accepted September 19, 1997)