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Increased sensitivity of transmitter release to calcium in ethanol tolerance

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In vitro treatment with ethanol is known to inhibit depolarization-induced release of neurotransmitters [1, 2]. In contrast, ethanol treatment *in vivo*, resulting in the development of ethanol tolerance, produced preparations which showed increased transmitter release compared to controls [1–4]. In the case of [^{3}H]DA release from striatal slices this increase was obtained after acute or chronic ethanol treatment, and during the first 12 hr of ethanol withdrawal, whether release was stimulated by 40 mM KCl or the Ca^{2+} ionophore A23187 [5]. The mechanism responsible for these alterations in release characteristics produced by the administration of ethanol *in vivo* is unknown but the latter findings suggest that some increase in the ability of Ca^{2+} entry to elicit neurotransmitter release may have occurred. In order to examine this possibility striatal slices were prepared from control and ethanol-tolerant animals and A23187-induced release studied at concentrations of Ca^{2+} ranging from 5 μM to 3 mM. In addition, it is now well established that mitochondria [6] and endoplasmic reticulum [7] are involved in control of intracellular Ca^{2+} concentration and so it was considered important to investigate the Ca^{2+} storage capacity of these subcellular organelles in preparations from control and ethanol-tolerant animals. If more Ca^{2+} is stored in a releasable pool in ethanol-tolerant brains, an increase in intraterminal Ca^{2+} concentration might originate from these storage sites. Therefore caffeine, which allows release of Ca^{2+} from endoplasmic reticulum [8] and Ruthenium Red (RR) and dinitrophenol (DNP), which allow release of Ca^{2+} from mitochondria [9, 10], were examined for their stimulatory effect on [^{3}H]-DA release, in preparations from control and ethanol-treated rats. It should be possible to establish, from the results of the present experiments, whether there is an increase in sensitivity of the release process to Ca^{2+} associated with ethanol tolerance and/or whether there is a change in the ability of mitochondria or endoplasmic reticulum to sequester Ca^{2+}.

Materials and methods

Materials. [^{3}H]Dopamine (sp. act. 41 mCi/mmol, 98% radiochemically pure) was obtained from Amersham, U.K. A23187 was obtained from Sigma Chemical Co., as was the enzyme assay kit for measurement of blood ethanol.

Animal treatments. Male Sprague–Dawley rats (250–350 g) were randomly divided into an ethanol treatment group, which was exposed to ethanol vapour for 6 or 7 days as previously described [5], or a control group, which was kept under identical conditions but without ethanol. Rats receiving ethanol inhaled ethanol vapour in increasing concentrations, from 10 mg/l in the first two days to approximately 20 mg/l at the end of the treatment period. It has been shown that under these conditions, tolerance (i.e. functional adaptation to the presence of ethanol) develops rapidly [11]. Animals were killed by stunning and decapitation, arterio-venous blood collected for analysis of ethanol concentration using an enzyme assay kit (Sigma Chemical Co.).

Tissue preparation. Brains were rapidly removed, the striatum dissected out and slices (0.2 mm thick) prepared by chopping the tissue in two directions using a McIlwain tissue chopper. Slices were placed in 3 ml oxygenated Krebs solution containing 5 μl [^{3}H]dopamine ([^{3}H]DA; final concentration 4×10^{-5} M), incubated for 30 min at 37° and continuously bubbled with 95% O_2/5% CO_2.

Procedure for studying release of [^{3}H]DA. Release of [^{3}H]DA was studied using the filtration method previously described for investigating release of ^{14}C-glutamate [12], with slight modifications. Briefly, aliquots of tissue suspension were placed on filters (0.45 μm pore size) in a Millipore filtration manifold and rinsed under vacuum with oxygenated Krebs solution to remove excess radiolabel. Slices were then incubated in 1 ml Krebs solution for 5 min and the filtrate was discarded. This procedure was repeated and the filtrate retained for estimation of basal [^{3}H]DA release. In the first series of experiments, slices were incubated in 1 ml Krebs solution containing A23187 (250 μM). The concentration of Ca^{2+} in the stimulating solution varied between 5 μM and 3 mM. In the second series of experiments, release was stimulated by incubating the slices for 5 min in 1 ml Krebs solution containing KCl (40 mM), dinitrophenol (DNP, 100 μM), Ruthenium Red (RR, 10 μM) or caffeine (10 mM). In all cases, filtrate was retained for scintillation counting. In both experiments, a final incubation in Krebs solution was carried out to ensure that release of [^{3}H]DA had returned to near basal levels. Aliquots (0.25 ml) of filtrates and the tissue-loaded filter papers were added to 5 ml scintillant (Beckman EP) and [^{3}H]DA was counted for 2 min. Total tissue accumulation was calculated by adding the radioactivity present in the filtrate after each incubation period to that remaining on the filter papers. The [^{3}H] released during each incubation period was then expressed as a percentage of the total amount present at the start of that particular incubation.

Statistical analysis. The Student's *t*-test was used to evaluate the data. The level of significance was chosen at $P < 0.05$.

Results and discussion

Mean blood ethanol concentration in the tolerant animals was 21.59 mM (±2.67, S.E.M.) in the first series of experiments and 20.01 (±2.82) in the second. Ethanol concentrations in the control animals were no higher than blanks.

In the first series of experiments, mean accumulation of [^{3}H]DA into the tissue slices was similar in control and ethanol-tolerant preparations, confirming our previous finding [5]. Similarly, there was no significant difference between mean basal release from control and ethanol-tolerant preparations. Figure 1 shows the effect of A23187 on release of [^{3}H]DA (with basal release subtracted) at several concentrations of Ca^{2+}. The relationship between release and log [Ca^{2+}] was found to be linear with regression coefficients of 0.97 and 0.96 in the control and ethanol-tolerant groups respectively. The corresponding slopes were 0.80 and 1.65. At each individual [Ca^{2+}], release from slices prepared from ethanol-tolerant rats was greater than the corresponding control value, reaching significance level in six out of eight cases ($^*P < 0.05$; $^{**}P < 0.01$). The results of the present experiments therefore firstly confirm our earlier findings, that release of [^{3}H]DA from striatal slices prepared from animals which had been made tolerant to ethanol *in vivo* was significantly enhanced compared to slices prepared from control animals [2, 5]. Secondly, in preparations from ethanol-tolerant animals, there appears to be an increase in the sensitivity of the release process to Ca^{2+}. Ca^{2+} influx into the presynaptic terminal is followed by a series of events leading to transmitter release, many of which are dependent on the presence of Ca^{2+} and/or the

Ca^{2+}-binding protein, calmodulin, e.g. activation of protein kinases, synaptic protein phosphorylation, vesicle–membrane interaction [13–16]. Thus the increase in sensitivity to Ca^{2+} reported here may reflect a change in any one or combination of these steps, or a change in the concentration of calmodulin or affinity of calmodulin for Ca^{2+}. Clearly, further experiments are required to pinpoint the nature of the increase in Ca^{2+} sensitivity. However, previously we reported that [^{3}H]NA release from synaptic vesicles, prepared from cerebral cortex of ethanol-tolerant rats, was unchanged [17] despite the fact that release from slices was increased [4]. This probably indicates that vesicle–membrane interactions are unlikely to be responsible for any increase in sensitivity to Ca^{2+}.

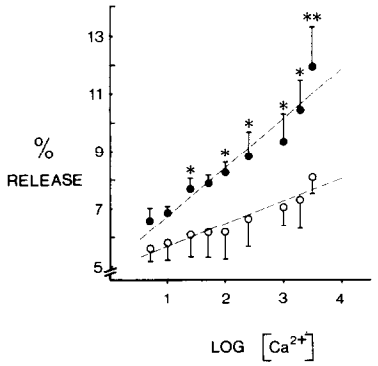

Fig. 1. A23187-induced release of [^{3}H]DA from striatal slices prepared from control (○—○) and ethanol-tolerant (●—●) rats. Animals received ethanol by inhalation for 6 or 7 days, while control animals were kept under identical conditions but received no ethanol. Mean blood ethanol concentration was 21.59 mM (±2.67, S.E.M.; N = 9) in the treated group and no higher than blanks in the control group (N = 8). Results given are the mean (±S.E.M.) release of [^{3}H]DA expressed as a percentage of the total radiolabel present at the start of the incubation period, minus basal release, at nine different concentrations of Ca^{2+}. Mean basal release was 5.41% (±0.175, S.E.M.) in the control group and 5.48% (±0.137) in the ethanol-treated group. The relationship between log [Ca^{2+}] and release approaches linearity with regression coefficients of 0.96 and 0.97 in the treated and control groups. Release of [^{3}H]DA from ethanol-tolerant preparations was higher than the corresponding control value at all Ca^{2+} concentrations, reaching statistical significance when marked with asterisks (*$P < 0.05$; **$P < 0.01$).

When control tissue preparations are treated with ethanol *in vitro*, there is an enhancement of A23187-induced release of [^{3}H]DA and it has been suggested that this change is due to the increase in mobility of the ionophore in the more fluid membrane associated with *in vitro* exposure to ethanol [18]. In the present study, however, the enhanced release of [^{3}H]DA in ethanol tolerance is not likely to be a consequence of the change in synaptic membrane composition which accompanies the development of ethanol tolerance [19, 20], since membrane fluidity decreases, probably impeding rather than facilitating the mobility of the ionophore. Although it is possible that the present increase in release of [^{3}H]DA could result from an increase in metabolism or uptake of dopamine in ethanol-tolerant preparations, this is probably also unlikely because there is no evidence of a change in metabolism or concentration [21] in ethanol tolerance and the present findings indicate that total tissue accumulation, reflecting uptake of the radiolabel, was similar in control and ethanol-tolerant preparations.

In the second series of experiments, the effect of KCl, DNP, RR and caffeine were examined on basal release of [^{3}H]DA. Mean accumulation and basal release of [^{3}H]DA were similar in control and ethanol-tolerant preparations and in all treatment groups. Release was significantly increased above basal levels when slices were incubated in the presence of any of the stimulating agents ($P < 0.01$; results not shown), but as shown in Fig. 2, the effect of 40 mM KCl was several times greater than the effect of the other agents. This was not surprising since KCl resulted in depolarization of the membrane, allowing the influx of Ca^{2+} from the incubating medium ([Ca^{2+}] = 2 mM) into the presynaptic terminal, while RR and DNP simply allow release of stored Ca^{2+} from mitochondria [7, 9] and caffeine release of stored Ca^{2+} from endoplasmic reticulum [8] (where the concentration of Ca^{2+} are probably orders of magnitude less [7]). In the present experiments, K^+-induced release of [^{3}H]DA from ethanol-tolerant preparations was significantly greater than from control prep-

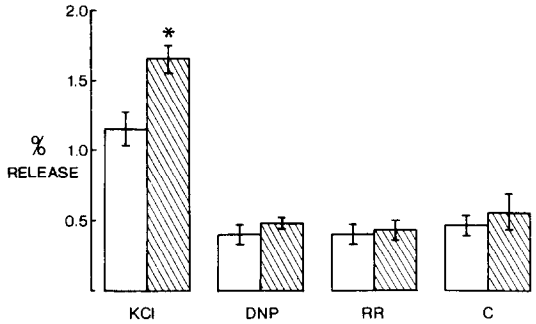

Fig. 2. Results presented are the mean (±S.E.M.) release of [^{3}H]DA stimulated by KCl (40 mM), dinitrophenol (DNP, 100 μM), Ruthenium Red (RR, 10 μM) or caffeine (C, 10 mM). Mean basal release was similar in control and ethanol-treated preparations and in the four treatment groups. The overall mean was 6.11% (±0.49, S.E.M.) in the control-treated groups and 6.09% (±0.32) in the ethanol-treated groups. In each case basal release was subtracted and stimulated release expressed as a percentage of the total radiolabel present at the start of the incubation period. KCl-induced release from ethanol-tolerant preparations (hatched histograms, N = 9) was significantly greater than from control preparations (*$P < 0.01$; plain histograms, N = 4). Release induced by any of the other agents was significantly greater than basal values (results not shown) but there was no difference between stimulated release in control and ethanol-tolerant preparations.

arations (Fig. 2; $P < 0.01$), confirming our earlier findings [2, 5]. However, release stimulated by DNP, RR or caffeine was similar in both preparations, suggesting that ethanol tolerance was not likely to be associated with an increase in the capacity of either mitochondrial or endoplasmic reticulum to store Ca^{2+}. It should be pointed out that we did not measure the concentration of intracellular Ca^{2+} following *in vitro* treatments but evidence from previous experiments has indicated that caffeine, DNP and RR all induce an increase in Ca^{2+} concentration [8–10], resulting in increased transmitter release from synaptosomes [9] or slices [22].

Our results suggest that in ethanol tolerance, enhanced [^{3}H]DA release from striatal slices results from an increase in the sensitivity of the release process to Ca^{2+} and not from an increase in capacity of intracellular stores to sequester and probably later release Ca^{2+}. This does not rule out a role for stored Ca^{2+} in the enhanced release of neurotransmitter, however, since it has been reported that inositol phosphate production on depolarization is enhanced in preparations from ethanol-tolerant animals [23, 24]. Inositol triphosphate has been shown to release intracellular Ca^{2+} from a variety of cell types [25, 26] so that its increased production in the ethanol-treated preparations may contribute to enhanced neurotransmitter release.

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Recognition of muscarinic acetylcholine receptor ligands by monoclonal antibodies against propylbenzilylcholine mustard

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Muscarinic acetylcholine receptors are involved in neurotransmission in the autonomic and central nervous systems. Ligand binding studies indicate that these receptors are composed of two subclasses [1]. Some muscarinic ligands can discriminate between the receptor subclasses, and binding of these ligands to a portion of the total receptor population results in selective biochemical and physiological responses [1–4]. It is likely that detailed classification of muscarinic ligands and accurate description of the ligand binding properties of the receptor will lead to the identification of selective drugs with enhanced therapeutic usefulness.

In this report we describe the production of two monoclonal antibodies directed against propylbenzilylcholine mustard (PrBCM*), the affinity alkylating muscarinic antagonist [5]. These antibodies selectively recognized muscarinic, but not nicotinic, cholinergic ligands and may be used for obtaining information concerning the specificity of muscarinic ligands as well as the binding and biochemical properties of the receptor itself.

Materials and methods

Materials. Propylbenzilylcholine mustard and [^{3}H]quinuclidinyl benzilate (35.5 Ci/mmole) were from the Amersham Corp. BSA, *p*-nitrophenyl phosphate and cholinergic drugs were from Sigma, except for quinuclidinyl benzilate (Hoffmann-LaRoche Laboratories), which was a gift from W. A. Catterall, and McNA343 and pirenzipine, which

* Abbreviations: BSA, bovine serum albumin; ELISA enzyme-linked immunosorptive assay; PrBCM, propylbenzililylcholine mustard; and QNB, quinuclidinyl benzilate.