Ethanol excitation of dopaminergic ventral tegmental area neurons is blocked by quinidine

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Abbreviations: aCSF, artificial cerebrospinal fluid; AHP, afterhyperpolarization; BK, large conductance calcium-dependent potassium current; DA, dopaminergic; \( I_h \), hyperpolarization-activated cationic current; SK, small conductance calcium-dependent potassium current; TEA, tetraethylammonium; VTA, ventral tegmental area;

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ABSTRACT

The dopaminergic (DA) neurons in the ventral tegmental area (VTA) are important for the reinforcing effects of ethanol. We have shown that ethanol directly excites DA VTA neurons and reduces the afterhyperpolarization (AHP) which follows spontaneous action potentials in these neurons. These data suggested that ethanol may be increasing the firing rate of DA VTA neurons by modulating currents which contribute to the AHP, either by reducing a K+ current or by increasing the inward current I\_h. In the present study, different blockers of K+ channels and I\_h were tested to determine if any could prevent the ethanol excitation of DA VTA neurons. Extracellular single-unit recordings and whole cell patch clamp recordings were made from DA VTA neurons in brain slices from Fischer 344 rats and ethanol (40 - 120 mM) and channel blockers were applied in the bath. Ethanol excitation was not reduced by blockade of I\_h with cesium (5 mM) or ZD7288 (30 \mu M), or by block of G-protein-coupled inwardly rectifying K+ channels (GIRKs) with barium (500 \mu M). Tetraethylammonium ion (TEA, 2 - 10 mM) which blocks the BK type calcium-dependent K+ current and some types of delayed rectifier currents, had no effect on the ethanol-induced excitation. Interestingly, ethanol excitation of DA VTA neurons was blocked by quinidine (20 - 80 \mu M), a drug which blocks many types of delayed rectifier K+ channels, including some insensitive to TEA. This effect of quinidine was concentration-dependent and reversible. These results suggest that ethanol excites DA VTA neurons by reducing a quinidine-sensitive K+ current.
Dopaminergic (DA) neurons in the VTA are the cells of origin of the mesolimbic dopamine pathway and provide the DA innervation to the nucleus accumbens (Oades and Halliday, 1987). Behavioral and pharmacological data support the involvement of the mesolimbic dopamine pathway in the reinforcing effects of ethanol. Both oral self-administration and systemic injection of ethanol increase dopamine release in the nucleus accumbens (Di Chiara and Imperato, 1988; Weiss et al., 1993). Pharmacological studies with DA agonists and antagonists also support the involvement of dopamine in ethanol reinforcement (Samson et al., 1990). Interestingly, rats will self-administer ethanol directly into the VTA (Rodd-Henricks et al., 2000). Ethanol excites dopamine cell bodies in the VTA in vivo in unanesthetized rats (Gessa et al., 1985) which would result in release of dopamine from terminals in the nucleus accumbens. We have shown that ethanol also excites DA VTA neurons in brain slices and that this effect persists when Ca\(^{2+}\)-dependent synaptic transmission is blocked with low Ca\(^{2+}\)/high Mg\(^{2+}\) media (Brodie et al., 1990). Furthermore, we have demonstrated that behaviorally reinforcing concentrations of ethanol excite acutely dissociated DA VTA neurons which have been isolated from all synaptic inputs, providing conclusive evidence that ethanol directly excites DA VTA neurons (Brodie et al., 1999b).

The present study addresses the question of the mechanism by which ethanol directly excites DA VTA neurons. Our previous intracellular studies in brain slices have shown that ethanol reduces the afterhyperpolarization (AHP) which follows spontaneous action potentials in DA VTA neurons (Brodie and Appel, 1998) which suggests that ethanol may be increasing the firing rate of DA VTA neurons by reduction of a K\(^{+}\) current which contributes to the AHP. To test this hypothesis, we have used a number of different K\(^{+}\) channel blockers to determine if any of these could prevent the ethanol excitation of DA VTA neurons. One current which contributes to the AHP in DA neurons
is the SK type calcium-dependent K⁺ current (Shepard and Bunney, 1991). In a previous study, we found that blockade of SK with apamin did not block ethanol excitation (Brodie et al., 1999a). In fact, SK blockade potentiated the ethanol excitation. These data indicate that the ethanol excitation of DA VTA neurons is not due to a reduction of SK current.

Several other types of K⁺ currents have been demonstrated in DA neurons (Silva et al., 1990) which could also contribute to the AHP. These include the BK type calcium-dependent K⁺ current and a delayed rectifier current, which are both blocked by tetraethylammonium ion (TEA) (Silva et al., 1990). In the present study, TEA was used to determine whether block of these two types of K⁺ currents could prevent ethanol excitation of DA VTA neurons.

At least 5 gene families (Kv1, Kv2, Kv3, eag and KCNQ) have members which code for K⁺ channels of the delayed rectifier type (Coetzee et al., 1999). Quinidine is a drug which blocks many types of delayed rectifier K⁺ currents from these 5 gene families (Yeola et al., 1996; Snyders et al., 1992; Singh and Singh, 1999; Rettig et al., 1992; Schonherr et al., 2002; Kang et al., 2001), including some that are insensitive to TEA (Grissmer et al., 1994). Therefore, the effect of quinidine on ethanol excitation of DA VTA neurons was also tested in the present study.

In addition, DA neurons have G-protein-coupled inwardly rectifying K⁺ channels (GIRKs) (Kim et al., 1995) and the VTA shows high levels of GIRK2 mRNA expression (Karschin et al., 1996). GIRK currents can be blocked by external barium ion (Werner et al., 1996). Since modulation of GIRK channels by ethanol has been recently reported (Lewohl et al., 1999; Kobayashi et al., 1999), the present study examined GIRK channel involvement by determining the effect of barium on ethanol excitation of DA VTA neurons.
DA VTA neurons also have a hyperpolarization-activated inward rectifier current called (I_h) (Mueller and Brodie, 1989; Lacey et al., 1989). I_h can be blocked by external cesium ion or the more selective blocker ZD7288 (Mercuri et al., 1995; Harris and Constanti, 1995; Neuhoff et al., 2002). Our previous intracellular studies demonstrated that ethanol enhances I_h in some DA VTA neurons (Brodie and Appel, 1998). Since I_h is an inward current and could contribute a depolarizing influence during the AHP to shorten the interspike interval, we hypothesized that enhancement of I_h might contribute to ethanol excitation of DA VTA neurons. Therefore, the present study tested the effect of blockade of I_h with cesium or ZD7288 on the ethanol excitation of DA VTA neurons.
METHODS

**Preparation of brain slices.** Brain slices containing the ventral tegmental area (VTA) were prepared from male Fischer 344 rats (90 - 150 gm) as previously described (Brodie et al., 1999a). Animals used in this study were treated in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and all experimental methods were approved by the Animal Care Committee of the University of Illinois at Chicago. Briefly, following rapid removal of the brain, the tissue was blocked coronally to contain the VTA and substantia nigra; the cerebral cortices and a portion of the dorsal mesencephalon were removed. The tissue block was mounted in the vibratome and submerged in chilled artificial cerebrospinal fluid (aCSF). Coronal sections (400 μm thick) were cut and the slice was placed onto a mesh platform in the recording chamber. The slice was totally submerged in aCSF maintained at a flow rate of 2 ml/min; the temperature in the recording chamber was kept at 35°C. The composition of the aCSF in these experiments was (in mM): NaCl 126, KCl 2.5, NaH2PO4 1.24, CaCl2 2.4, MgSO4 1.3, NaHCO3 26, glucose 11. The aCSF was saturated with 95% O2/5% CO2 (pH=7.4). Equilibration time of at least one hour was allowed after placement of tissue in the recording chamber before electrodes were placed in the tissue.

**Cell identification.** The VTA was clearly visible in the fresh tissue as a grey area medial to the darker substantia nigra, and separated from the nigra by white matter. Recording electrodes were placed in the VTA under visual control. Dopamine neurons have been shown to have electrophysiological characteristics very different from non-dopaminergic neurons in the mesencephalon (Lacey et al., 1989). Only those neurons which were anatomically located within the VTA and which conformed to the criteria for dopaminergic neurons established in the literature and in this laboratory (Mueller and Brodie, 1989; Lacey et al., 1989) were studied. These criteria
include broad action potentials, slow spontaneous firing rate (0.5 - 5 Hz) with a regular interspike interval, and the presence of time-dependent inward rectification due to the presence of the hyperpolarization-activated cationic current (I_h).

**Drug Administration.** Drugs were added to the aCSF by means of a calibrated infusion pump from stock solutions 100 to 1000 times the desired final concentrations. The addition of drug solutions to the aCSF was performed in such a way as to permit the drug solution to mix completely with aCSF before this mixture reached the recording chamber. Final concentrations were calculated from aCSF flow rate, pump infusion rate and concentration of drug stock solution. The small volume chamber (about 300 μl) used in these studies permitted the rapid application and washout of drug solutions. Typically drugs reach equilibrium in the tissue after 2 to 3 minutes of application.

A stock solution of 95% ethanol (v/v USP) was used in the pump, and infusion of ethanol never exceeded 1% of the flow rate of the aCSF. Ethanol was administered for 6-7 min to ensure that measurements were made after the full ethanol concentration was reached in the tissue and the peak drug effect was attained.

The behaviorally active range for blood ethanol concentrations in the rat extends from 40 mM (sedation) to 90 mM (loss of righting reflex) (Majchrowicz and Hunt, 1976); the lethal blood ethanol concentration in rats is about 200 mM (LD50 = 202 mM) (Haggard et al., 1940). Rats will self-administer 44 - 55 mM ethanol directly into the VTA, indicating that this concentration is reinforcing in the whole animal (Rodd-Henricks et al., 2000). The present study examined ethanol concentrations in the range of 40 to 120 mM, pharmacologically relevant, sublethal concentrations in the rat.
Quinidine sulfate was purchased from Research Biochemicals International (Natick, MA) and ZD7288 was purchased from Tocris (Ellisville, Mo). Tetraethylammonium chloride (TEA) and cesium chloride were purchased from Sigma (St. Louis, MO) and barium chloride was purchased from Fisher Scientific (Fair Lawn, New Jersey).

**Extracellular recording.** Extracellular recording electrodes were made from 1.5 mm diameter glass tubing with filament and were filled with 0.9% NaCl. Tip resistance of the microelectrodes ranged from 4 - 8 MΩ. The Fintronics amplifier used in these recordings includes a window discriminator, the output of which was fed to both a rectilinear pen recorder, and a computer-based data acquisition system which was used for on-line and off-line analysis of the data. The multiplexed output of the Fintronics amplifier was displayed on an analog storage oscilloscope, for accurate adjustment of the window levels used to monitor single units. An IBM-PC-based data acquisition system was used to calculate, display and store the frequency of firing over 5 second and 1 minute intervals. Firing rate was determined before and during drug application. Firing rate was calculated over a 1 minute interval immediately prior to drug administration and a 1 minute interval during the peak drug effect; drug-induced changes in firing rate were expressed as the percentage change from the control firing rate according to the formula ((FR_D - FR_C) / FR_C) X 100, where FR_D is the firing rate during the peak drug effect and FR_C is the control firing rate. The change in firing rate thus is expressed as a percentage of the initial firing rate, which controls for small changes in firing rate which may occur over time.

**Whole-cell recording.** Patch electrodes were pulled from LE16 glass capillaries (Dagan, Corp, Minneapolis, MN) and the tips were fire-polished. Electrodes had resistances of 3 - 5 MΩ when filled with a solution containing (in mM): potassium gluconate 125, NaCl 15, CaCl₂ 1, MgCl₂
2, Hepes 10, EGTA 11, ATP 3, GTP 0.3, adjusted to pH 7.3 with TRIZMA base. The electrode was advanced into the brain slice and the extracellular spontaneous action potentials of DA VTA neurons were monitored in current clamp mode. Recordings were made with conventional whole cell patch-in-the-slice methodology. Voltage clamp and current clamp recordings were accomplished with an Axopatch-1B or Axoclamp-2A amplifier (Axon Instruments, San Rafael, CA). Current and voltage were monitored on a storage oscilloscope and on a rectilinear pen recorder and recorded on an on-line PC computer. Current and voltage steps, data acquisition and data analysis were controlled with pClamp 8 software (Axon Instruments, Foster City, CA).

**Statistical analysis.** Averaged numerical values were expressed as the mean ± the standard error of the mean (S.E.M.). The significance of firing rate changes before and after a single drug concentration was assessed with a paired t-test. For effects of multiple drug concentrations or more than one drug, an appropriate one- or two-way analysis of variance (ANOVA) was used, followed by Student Newman-Keuls post hoc comparisons when needed. In addition, the Bonferroni correction was used to determine the cutoff values for statistical significance of the data with 40 μM quinidine shown in Figs. 7 and 8, since the data in these two figures were obtained from the same neurons. Statistical analyses were performed with SigmaStat (SPSS, Chicago, IL).
RESULTS

Blockade of $I_h$ with cesium or ZD7288 does not reduce ethanol excitation of DA VTA neurons. Dopaminergic VTA neurons have a hyperpolarization-activated, time-dependent, cationic inward rectifier current called $I_h$ (Mueller and Brodie, 1989) which is activated by membrane hyperpolarization negative to about -70 mV. Figure 1A shows whole-cell voltage clamp recording of $I_h$. Part a, shows the family of hyperpolarizing voltage steps (duration 1 sec) from a holding potential of -60 mV to -140 mV (in 10 mV increments) used to evoke $I_h$. Parts b and c show current responses to this voltage protocol measured in control (b) and after bath application of 5 mM cesium (c) in the same DA VTA neuron. The amplitude of $I_h$ was measured as the difference between the instantaneous current measured just after the decay of the capacitive transient, and the steady-state current near the end of the voltage step. Figure 1B shows Current/Voltage (I/V) curves constructed for $I_h$ measured in control and after bath application of 5 mM cesium from the experiment shown in A. Figures 1A and 1B illustrate that $I_h$ in DA VTA neurons is completely blocked by bath application of 5 mM cesium. $I_h$ was completely blocked by 5 mM cesium in all such neurons that we have tested ($n = 8$) (Liu et al., 1999) and therefore this concentration of cesium was used to determine whether blockade of $I_h$ would alter the ethanol-induced excitation of DA VTA neurons.

Extracellular single unit recording was used to measure the effect of ethanol (40, 80 and 120 mM) on the firing rate of DA VTA neurons in brain slices. After washout of the last concentration of ethanol, cesium (5 mM) was added to the superfusate, and the same concentrations of ethanol were tested again. The percent increase in firing rate produced by each ethanol concentration was calculated with the formula shown in the Methods section. Figure 1C shows the pooled concentra-
tion-response curves for ethanol excitation measured in the absence and presence of 5 mM cesium in 6 DA VTA neurons. Ethanol produced a concentration-dependent increase in firing rate (two-way ANOVA, F = 15.08; df = 2, 20; p < 0.001). In the presence of cesium, the ethanol-induced excitation was not blocked, but was significantly greater than before cesium administration (F = 8.12; df = 1, 20; p = 0.01). Application of cesium (5 mM) alone significantly increased the mean spontaneous firing rate of these neurons (paired t-test, t = -5.94, df = 5, p = 0.002). Specifically, the mean firing rate was 1.58 ± 0.22 Hz in control and 3.52 ± 0.45 Hz in cesium (n = 6).

ZD7288 is a more selective blocker of I_h and 30 μM ZD7288 has been shown to completely block I_h in DA VTA neurons (Neuhoff et al., 2002). Therefore, the effect of ethanol (40, 80 and 120 mM) on the firing rate of DA VTA neurons was also assessed in the presence of ZD7288. Figure 2 shows the pooled concentration-response curves for ethanol excitation measured in the absence and presence of ZD7288 (30 μM) in 10 DA VTA neurons. The percent increase in firing rate produced by each ethanol concentration was calculated with the formula shown in the Methods section. Ethanol produced a concentration-dependent increase in firing rate (two-way ANOVA, F = 5.18; df = 2, 28; p = 0.012). ZD7288 had no significant effect on ethanol-induced excitation of DA VTA neurons (p > 0.05). Application of ZD7288 (30 μM) alone caused a small but significant decrease in the spontaneous firing rate of these neurons (paired t-test, t = 3.62, df = 9, p = 0.006). Specifically, the mean firing rate was 1.23 ± 0.09 Hz in control and 1.04 ± 0.07 Hz in ZD7288 (n = 10).

**Tetraethylammonium (TEA) does not block ethanol excitation of DA VTA neurons.**

TEA blocks both the BK type of the calcium-dependent K^+ current and some types of delayed
rectifier K\(^+\) currents (Coetzee et al., 1999). Therefore, TEA was used to determine whether block
of these two types of K\(^+\) currents would prevent ethanol excitation of DA VTA neurons. Figure 3A
illustrates the effect of TEA on action potential shape in a typical DA VTA neuron; this current
clamp recording was obtained with the whole cell patch-in-the-slice method. TEA (2 mM) increased
action potential duration and decreased the afterhyperpolarization (n = 3); 10 mM TEA caused
similar, but larger changes in spike shape (n = 3, data not shown).

Extracellular single unit recording was used to measure the effect of ethanol (40, 80 and 120
mM) on the firing rate of 11 DA VTA neurons in brain slices. After washout of the last
concentration of ethanol, TEA (2 mM) was added to the superfusate and the same concentrations
of ethanol were tested again in the continued presence of TEA. In 5 of these neurons, ethanol
responses were also measured again after 10 mM TEA administration. The percent increase in firing
rate produced by each ethanol concentration was calculated with the formula shown in the Methods
section. Figure 3B shows the pooled concentration-response curves for ethanol excitation measured
in the absence and presence of 2 and 10 mM TEA in these neurons. Ethanol produced a
concentration-dependent increase in firing rate (two-way ANOVA, F = 4.25; df = 2, 62; p < 0.02);
TEA had no significant effect on ethanol-induced excitation of DA VTA neurons (p > 0.05).
Application of TEA alone (2 mM or 10 mM) caused increases or decreases in the basal firing rate
of some DA VTA neurons, but on average there was no significant change in the basal firing rate.
Specifically, the mean basal firing rate was 1.70 ± 0.32 Hz and 1.81 ± 0.37 Hz in control and 2 mM
TEA, respectively (n = 11, paired t-test, p > 0.05), and 1.42 ± 0.26 Hz and 1.60 ± 0.31 Hz in control
and 10 mM TEA, respectively (n = 5, paired t-test, p > 0.05).
Barium does not block ethanol excitation of DA VTA neurons. Dopamine binding to D₂ autoreceptors on DA neurons activates G-protein-coupled inwardly rectifying K⁺ channels (GIRKs) (Kim et al., 1995). GIRK currents can be modulated by ethanol and blocked by external barium (see Introduction). Therefore, barium was used to determine whether GIRK channels contribute to the ethanol-induced excitation of DA VTA neurons. Figure 4, A and B, illustrate a ratemeter record indicating firing rate of a typical DA VTA neuron versus time. Prior to the addition of barium to the superfusate, ethanol (40, 80, and 120 mM) produced concentration-dependent excitation and dopamine (5 μM) produced a large inhibition in firing rate (Fig. 4A). In the presence of 500 μM barium (Fig. 4B), the effect of ethanol was similar, but the dopamine-induced inhibition was almost completely blocked (see legend). A similar block of inhibition by dopamine (5-10 μM) was seen in all cells tested (n = 6). The percent increase in firing rate produced by each ethanol concentration was calculated with the formula shown in the Methods section. Figure 4C shows the pooled concentration-response curves for mean percent increase in firing rate produced by ethanol in the absence and presence of 500 μM barium, from experiments in 9 DA VTA neurons, similar to that shown in Fig. 4, A and B. Ethanol produced a concentration-dependent increase in firing rate (two-way ANOVA, F = 22.86; df = 2, 36; p < 0.001). As can be seen on Fig. 4C, barium did not affect the response to 40 or 80 mM ethanol, but did appear to increase the excitation by 120 mM ethanol; the overall F value for the effect of barium on the two-way ANOVA was not significant (p > 0.05), but a Student-Neuman-Keuls post hoc comparison showed that the increased excitation by 120 mM ethanol in barium was significant (p < 0.05). Application of barium (500 μM) alone significantly increased the mean spontaneous firing rate of these neurons (paired t-test, t = -15.01, df = 8, p < 0.001). Specifically, the mean firing rate was 1.32 ± 0.13 Hz in control and 3.62 ± 0.19 Hz in
Quinidine blocks ethanol excitation of DA VTA neurons. Quinidine is a drug which blocks many types of delayed rectifier K⁺ currents, including some that are insensitive to TEA (see Introduction). If the ethanol excitation of DA VTA neurons is due to reduction of a delayed rectifier K⁺ current, we reasoned that the ethanol excitation of these neurons might be blocked by quinidine.

Figure 5A is a whole-cell current clamp recording of averaged spontaneous action potentials recorded from a typical DA VTA neuron in control and after bath application of quinidine. Note that the afterhyperpolarization was decreased by both 20 and 40 μM quinidine, in a concentration-dependent manner. Quinidine (20 - 80 μM) reduced the afterhyperpolarization following the spontaneous action potential in all DA VTA neurons tested (n = 7). Specifically, the mean percent reduction in the peak amplitude of the afterhyperpolarization was -20.4 ± 5.6% (n = 4) in 20 μM quinidine and -41.1 ± 11.1% (n = 5) in 80 μM quinidine. Both the reduction in the peak afterhyperpolarization amplitude by 20 μM quinidine (paired t-test, t = 4.25, df = 3, p < 0.05) and by 80 μM quinidine (paired t-test, t = 2.84, df = 4, p < 0.05) were statistically significant.

A typical extracellular ratemeter recording of spontaneous firing rate of another DA VTA neuron is shown in Fig. 5B. Prior to addition of quinidine to the superfusate, ethanol produced a 25.3% excitation of this neuron. Quinidine (20 μM) had only a small effect on the basal firing rate, causing a slight increase in firing rate and some decrease in the regularity of firing. In the presence of quinidine, 80 mM ethanol increased the firing rate by only 7.8% in this neuron.

Figure 6A shows the pooled concentration-response curves for mean percent changes in firing rate induced by ethanol (40, 80 and 120 mM) in the absence and presence of 20 μM
quinidine in 6 DA VTA neurons (from experiments similar to that shown in Fig. 5B). In the absence of quinidine (control), ethanol caused the usual concentration-dependent increase in firing rate. Quinidine (20 μM) significantly decreased the excitation by ethanol (two-way ANOVA, F = 15.22; df = 1, 22; p < 0.001). Figure 6B shows the pooled concentration-response curves for ethanol-induced changes in firing rate in 11 DA VTA neurons tested in the absence and presence of 40 μM quinidine. Quinidine (40 μM) also significantly decreased the excitation of DA VTA neurons by ethanol (two-way ANOVA, F = 13.79; df = 1, 32; p < 0.001). The effect of 120 mM ethanol was also measured in the absence and presence of 80 μM quinidine in 7 DA VTA neurons. Ethanol (120 mM) alone produced a mean increase in firing rate of 26.5 ± 2.6 % in these neurons. In the presence of 80 μM quinidine, 120 mM ethanol caused a mean increase in firing rate of only 0.7 ± 11.0 %. This block of ethanol excitation by 80 μM quinidine was statistically significant (paired t-test, t = 2.64, df = 6, p = 0.039). Note that in the presence of quinidine, ethanol actually decreased the firing rate in some of the DA VTA neurons tested.

Application of quinidine (20, 40 or 80 μM) alone had no statistically significant effect on the mean spontaneous firing rate of DA VTA neurons. Specifically, the mean firing rates of neurons tested with quinidine alone were: 1.08 ± 0.12 Hz in control and 1.37 ± 0.15 Hz in 20 μM quinidine (n = 6, paired t-test, p > 0.05); 1.13 ± 0.14 Hz in control and 1.06 ± 0.12 Hz in 40 μM quinidine (n = 11, paired t-test, p > 0.05); and 1.12 ± 0.06 Hz in control and 1.07 ± 0.13 Hz in 80 μM quinidine (n = 7, paired t-test, p > 0.05). Although quinidine did not significantly change the mean firing rate, occasional bursting was observed in some neurons.

**Quinidine reduction of ethanol excitation is concentration-dependent and reversible.**
The percent change in firing rate produced by 80 mM ethanol was measured in 21 DA VTA neurons in control and again in the presence of quinidine (1 - 40 μM). Figure 7 is the pooled concentration-response curve for these experiments in which the mean percent change in firing rate produced by 80 mM ethanol is plotted as a function of the quinidine concentration present. Note that quinidine reduced the excitation produced by 80 mM ethanol in a concentration-dependent manner, with higher concentrations of quinidine causing larger reductions in the ethanol response. Quinidine had a significant effect on the magnitude of the response to 80 mM ethanol (one way ANOVA, F = 7.16; df = 5, 54; p < 0.001) and Student-Newman-Keuls post hoc comparisons indicated that ethanol responses in 10, 20 and 40 μM quinidine were significantly different from control ( p < 0.005 in all cases).

Note that the magnitude of ethanol excitation typically remains stable throughout the duration of the experiments (Brodie et al., 1990), which can last for two to three hours, so that the decrease in the effect of ethanol is due to the presence of quinidine, not to fading of the ethanol response with time. This was substantiated in experiments looking at reversal of the quinidine-induced reduction of the ethanol excitation upon washout of quinidine. The change in firing rate in response to 80 mM ethanol was measured in 5 DA VTA neurons before and then in the presence of 40 μM quinidine. Following blockade of the ethanol-induced excitation, quinidine administration was stopped, and 80 mM ethanol was tested subsequently at 10 minute intervals. Four of 5 neurons showed reversal of the quinidine effect within 45 minutes, and the fifth cell showed reversal after one hour of washout. Figure 8 illustrates that the effect of quinidine on ethanol excitation completely reverses upon washout of quinidine. Before quinidine, ethanol caused a mean increase in firing rate of 22.9 ± 3.2%. In the presence of 40 μM quinidine, the excitatory effect of
ethanol was blocked and the mean change in firing rate in response to ethanol was a small decrease (-1.7 ± 4.2%). Upon washout of quinidine, the mean ethanol-induced increase in firing rate was 23.7 ± 1.7%. A one-way repeated measures ANOVA indicated a significant effect of treatment condition (F = 24.64; df = 2, 8; p < 0.001) and Student-Newman-Keuls post-hoc comparisons indicated that the ethanol response in quinidine was significantly different from both the ethanol responses before quinidine and after washout (both p < 0.001), and that ethanol responses before quinidine and after washout did not differ (p > 0.05).
DISCUSSION

The present study demonstrates that quinidine can block the ethanol excitation of DA VTA neurons. To our knowledge, this is the first pharmacological agent found to block the direct effect of ethanol on DA VTA neurons. Of the various K⁺ channel blockers (TEA, barium, cesium and quinidine) and Iₕ channel blockers (cesium and ZD7288) tested in this study, only quinidine was able to decrease ethanol excitation of DA VTA neurons.

TEA blocks both the BK type of the calcium-activated K⁺ current and some types of delayed rectifier K⁺ currents (Coetzee et al., 1999; Silva et al., 1990). The present study shows that TEA (2 and 10 mM) prolonged action potential duration and reduced the afterhyperpolarization but did not block the ethanol excitation. These data indicate that the ethanol-induced excitation of DA VTA neurons is not due to a reduction in BK type calcium-activated K⁺ current or a TEA-sensitive delayed rectifier current.

Barium ion blocks G-protein-coupled inwardly rectifying K⁺ channels (GIRKs) (Werner et al., 1996). In the present study, barium (500 μM) blocked the inhibitory effect of dopamine, a GIRK-mediated response, but did not block the ethanol excitation. These data indicate that the ethanol-induced excitation of DA VTA neurons is not mediated by GIRK channels. The excitation produced by 120 mM ethanol was significantly increased in the presence of barium. This enhancement could be due to the fact that barium increases the membrane resistance (Lacey et al., 1987), which would tend to amplify the effect of changes in other currents. In addition, ethanol has been shown to enhance GIRK-mediated K⁺ currents in cerebellar neurons and to activate GIRK channels expressed in Xenopus oocytes (Lewohl et al., 1999; Kobayashi et al., 1999). If ethanol has
a similar effect in DA VTA neurons, this would be an inhibitory factor which could oppose the
direct excitatory action of ethanol which has been demonstrated to occur in these neurons (Brodie
et al., 1999b). Barium blockade of GIRK channels, therefore, might lead to enhanced net excitation
by ethanol.

The hyperpolarization-activated inward current $I_h$ is blocked by cesium ion and by ZD7288
(Mercuri et al., 1995; Harris and Constanti, 1995; Neuhoff et al., 2002). In the present study,
external cesium (5 mM) completely blocked $I_h$ in DA VTA neurons, but neither cesium, nor the
more selective blocker ZD7288, reduced the ethanol excitation of these neurons. These data indicate
that the ethanol-induced enhancement of $I_h$ which occurs in some DA VTA neurons (Brodie and
Appel, 1998) is not a major factor mediating ethanol excitation of these neurons.

Cesium did not block ethanol excitation of DA VTA neurons, but actually potentiated it.
In contrast, the more selective $I_h$ antagonist ZD7288 did not alter the size of the ethanol excitation,
suggesting that the potentiation of ethanol excitation by cesium was not due to block of $I_h$, but to
other actions of cesium. Like barium, mentioned above, cesium increases the membrane resistance
of DA VTA neurons (Mercuri et al., 1995) and can block GIRK channels (Lesage et al., 1995),
actions which could explain the potentiation of ethanol excitation seen with these agents. Cesium,
like barium, also increased the basal firing rate of DA VTA neurons by about two-fold. In contrast,
the more selective $I_h$ antagonist ZD7288 (30 μM) caused a small but significant decrease in
spontaneous firing rate. This observation is consistent with our preliminary data and data in the
literature showing that higher concentrations of ZD7288 cause large decreases in firing rate
(Neuhoff et al., 2002).
Our previous data suggested that ethanol excites DA VTA neurons by reduction of the AHP (Brodie and Appel, 1998). A number of different currents contribute to the AHP and we have examined the involvement of some of these current components in mediating the ethanol excitation. The present study excludes the involvement of $I_h$, the TEA-sensitive delayed rectifier $K^+$ current and the BK type calcium-dependent $K^+$ current. Previously, we showed that blockade of SK type calcium-dependent $K^+$ current with apamin or d-tubocurarine, did not block ethanol excitation of DA VTA neurons, excluding the involvement of this current (Brodie et al., 1999a).

The present study demonstrates that quinidine (20 - 80 $\mu$M) blocks the ethanol excitation of DA VTA neurons. The quinidine-induced reduction of ethanol excitation in these neurons was concentration-dependent over the range of 1- 80 $\mu$M and was fully reversible by 45 min to 1 hr after washout of quinidine. Similar concentrations of quinidine block delayed rectifier $K^+$ channel members from each of the 5 different gene families, specifically, Kv1 channels (Yeola et al., 1996; Snyders et al., 1992), Kv2 channels (Singh and Singh, 1999; Yeola et al., 1996), Kv3 (Rettig et al., 1992), eag channels (Schonherr et al., 2002) and KCNQ (KvLQT) channels (Kang et al., 2001). Our preliminary whole cell voltage clamp experiments show that ethanol (30 - 100 mM) reduces a sustained, delayed rectifier type of $K^+$ current in DA VTA neurons, both in acutely dissociated neurons and brain slices (Brodie et al., 2000). Taken together, these data suggest that ethanol excitation of DA VTA neurons may be due to reduction of a quinidine-sensitive, delayed rectifier $K^+$ current.

Our previous work has shown that ethanol excitation (Brodie et al., 1999b) and reduction of delayed rectifier current (Brodie et al., 2000) are direct effects on DA VTA neurons since they occur in acutely dissociated DA VTA neurons which have been stripped of all synaptic inputs. It
is likely, therefore, that quinidine blocks this direct ethanol excitation through an action on K+ channels on the DA VTA neurons themselves. This is further supported by the observation in the present study, that quinidine reduces the AHP of spontaneous action potentials in the DA VTA neurons. If GABAergic neurons exerted a tonic modulation of DA VTA neurons in the slice and quinidine altered the activity of GABAergic neurons, this would be expected to change the basal firing rate of DA neurons, but the present study shows that quinidine did not alter the basal firing rate of DA VTA neurons. Future experiments in acutely dissociated DA VTA neurons are planned which could verify that quinidine is acting on the DA VTA neurons themselves.

Quinidine effectively blocked the ethanol excitation, but did not, itself, cause a significant increase in firing rate. There are several reasons why quinidine may not exert the same actions on firing rate as ethanol and yet act on the same K+ channel to prevent ethanol’s effect. First, quinidine is not a selective agent and in addition to blocking a number of different delayed rectifier K+ channels (see above), it can also block other types of K+ channels (Yeola and Snyders, 1997; Rettig et al., 1992), Na+ channels and Ca2+ channels (Ducouret, 1976; Ragsdale et al., 1996) although depending on the preparation, these other actions may require higher concentrations of quinidine (Hermann and Gorman, 1984). It would be the sum of all the actions of quinidine on DA VTA neurons which would determine its net effect on firing rate. Ethanol could be acting on one type of K+ channel or a small subset of the channels blocked by quinidine. Second, at low concentrations, quinidine may bind to the channel and change its conformation, thereby allosterically preventing ethanol block of the channel. This action might occur at lower concentrations than required for quinidine itself to block the channel. Voltage clamp experiments on specific ionic currents will be necessary to understand how quinidine reduces the excitatory effect of ethanol. It is intriguing from
a therapeutic point of view that quinidine blocked the ethanol excitation of DA VTA neurons without exciting these neurons and therefore may not be reinforcing or addictive itself.

In summary, the pharmacological data we have previously published and the data presented in this paper indicate that the ethanol-induced excitation of DA VTA neurons is not mediated by a change in SK (Brodie et al., 1999a) or BK type calcium-activated K⁺ currents, GIRK currents, or the inward current Iᵢ. Our preliminary voltage clamp data and the data in the present study suggest that ethanol excitation of DA VTA neurons may be due to reduction of a quinidine-sensitive, delayed rectifier K⁺ current. Ongoing work in our laboratory is directed at identification of this native ethanol-sensitive K⁺ current in DA VTA neurons, in terms of cloned delayed rectifier K⁺ channels of known structure. Identification of this K⁺ channel protein, would point to the gene responsible for its expression. Such a discovery could have major implications for understanding genetic differences in ethanol effects on the mesolimbic reward pathway and how changes in the response of DA VTA neurons during chronic ethanol consumption leads to alcohol craving and addiction. The observation in the present study that quinidine can block ethanol excitation of DA VTA neurons is important in its own right, since DA VTA neurons are critical components of the central reward pathway underlying the reinforcing properties of ethanol. Quinidine could be a prototypic drug for blocking the reinforcing effects of ethanol, and therefore could point to a new target for development of therapeutic agents for the treatment of alcohol abuse.
REFERENCE LIST


Haggard HW, Greenberg LA and Rakieten N (1940) Studies on the absorption, distribution and elimination of alcohol. VI. The principles governing the concentration of alcohol in the blood and the concentration causing respiratory failure. *J Pharmacol Exp Ther* 69:252-265.


Lacey MG, Mercuri NB and North RA (1987) Dopamine acts on D2 receptors to increase potassium conductance in neurones of the rat substantia nigra zona compacta. *J Physiol (Lond)* 392:397-416.


Liu ZP, Appel SB and Brodie MS (1999) Serotonin reduces time-dependent inward rectification (Ih)


FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Cesium blocks $I_h$ but not ethanol-induced excitation of DA VTA neurons. A, Whole cell voltage clamp recording of $I_h$. a, Hyperpolarizing voltage steps used to elicit current responses shown in b and c, below. The instantaneous current was measured just after the decay of the capacitive transient and the steady-state current was measured near the end of the voltage command. b, Control currents. c, Currents measured after 5 mM cesium was added to the extracellular medium in the same DA VTA neuron. B, Current-voltage (I/V) curves of the hyperpolarization-activated inward current $I_h$ measured in control and after 5 mM cesium was added to the extracellular medium. $I_h$ was evoked in whole-cell voltage clamp mode by hyperpolarizing voltage steps from a holding potential of -60 mV as shown in A. The amplitude of $I_h$ was calculated by subtracting the instantaneous current from the steady-state current. Note that $I_h$ was completely blocked by 5 mM cesium (same neuron as in A). C, Pooled concentration-response curves for ethanol excitation measured in the absence and presence of 5 mM cesium in 6 DA VTA neurons. Spontaneous firing rate was measured with extracellular single unit recording and percent increases in firing rate produced by ethanol (40, 80, and 120 mM) were calculated with the formula shown in the Methods section. Error bars represent S.E.M. In the presence of cesium, ethanol-induced excitation was not blocked, but was significantly greater than before cesium administration (two-way ANOVA, $p = 0.01$). As usual, the ethanol excitation was concentration-dependent ($p < 0.001$).
Fig. 2. ZD7288, a more selective blocker of $I_h$, does not change ethanol-induced excitation. The effect of ethanol (40, 80 and 120 mM) on the firing rate of DA VTA neurons was measured with extracellular single unit recording in the absence and presence of ZD7288 (30 μM). Pooled concentration-response curves for ethanol excitation measured in control and in the presence of 30 μM ZD7288 in 10 DA VTA neurons. There was no statistically significant effect of ZD7288 on the ethanol concentration-response curve (two-way ANOVA, $p > 0.05$). The ethanol excitation was concentration-dependent ($p = 0.012$).

Fig. 3. Tetraethylammonium (TEA) alters action potential shape but not ethanol-induced excitation. A, Spontaneous action potentials from a typical DA VTA neuron measured with whole-cell patch, current clamp recording in a brain slice. Each trace represents the average of at least 20 action potentials collected in the same neuron over a 30 second period before TEA (control) or in the presence of 2 mM TEA. TEA increased the duration of the spontaneous action potential and decreased the amplitude of the afterhyperpolarization. B, Pooled concentration-response curves for ethanol (40, 80, and 120 mM) effects on firing rate measured in DA VTA neurons with extracellular single unit recording in the absence and presence of 2 mM TEA ($n = 11$) or 10 mM TEA ($n = 5$). There was no statistically significant effect of either concentration of TEA on the ethanol concentration-response curves (two-way ANOVA, $p > 0.05$). The ethanol excitation was concentration-dependent ($p < 0.02$).
**Fig. 4.** Barium blocks dopamine inhibition but not ethanol excitation of DA VTA neurons. A, Effect of ethanol and dopamine before barium administration. Extracellular single unit recording of the firing rate of a typical DA VTA neuron. Firing rate is plotted as a function of time; vertical bars are proportional to the mean firing rate over 5 sec intervals. Horizontal bars indicate duration of application of either ethanol (EtOH) or dopamine (DA). Bath application of ethanol produced concentration-dependent increases in firing rate of 6.1% (40 mM), 13.4% (80 mM), and 23.2% (120 mM). Application of dopamine (5 μM) decreased the firing rate of the same neuron by 88.6%. B, Continuation of the extracellular recording from the same cell as in part A, showing the effect of ethanol and dopamine applied in the presence of 500 μM barium. Barium alone produced a 144% increase in the basal firing rate. In barium, ethanol increased the firing rate of this cell by 11.9% (40 mM), 19.4% (80 mM), and 28.5% (120 mM). Dopamine inhibited the firing rate by only 8.7% when barium was present, a decrease in the inhibitory effect of dopamine of about 90%. C, Pooled concentration-response curves for ethanol excitation measured in the absence and presence of 500 μM barium in 9 DA VTA neurons. Ethanol produced a concentration-dependent increase in firing rate (two-way ANOVA, p < 0.001). Barium did not affect the response to 40 or 80 mM ethanol, but did appear to increase the excitation by 120 mM ethanol. The overall F value for the effect of barium in the two-way ANOVA was not significant (p > 0.05), but a Student-Neuman-Keuls post hoc comparison showed that the increased excitation by 120 mM ethanol in barium was significant (p < 0.05).
**Fig. 5.** Quinidine reduces the spike afterhyperpolarization and ethanol excitation. A, Whole-cell patch-in-the-slice, current clamp recording of spontaneous action potentials in a typical DA VTA neuron. Each trace is the average of at least 20 action potentials collected over a 30 second period before quinidine (control) or in the presence of 20 or 40 μM quinidine. Quinidine reduced the amplitude of the spike afterhyperpolarization in a concentration-dependent manner. B, Extracellular recording of the firing rate of another DA VTA neuron; vertical bars are proportional to the mean firing rate over 5 sec intervals. Horizontal bars indicate duration of application of 80 mM ethanol or 20 μM quinidine, as indicated. Prior to quinidine application, 80 mM ethanol produced a 25.3% increase in the firing rate of this neuron. In the presence of 20 μM quinidine, the magnitude of ethanol excitation was reduced to 7.8%.

**Fig. 6.** Quinidine blocks ethanol-induced excitation of DA VTA neurons. Pooled concentration-response curves show the effect of ethanol (40, 80, and 120 mM) on firing rate of DA VTA neurons, in the absence and presence of quinidine. A, Mean responses of 6 DA VTA neurons tested with ethanol before and in presence of 20 μM quinidine; error bars indicate S.E.M. Prior to quinidine, ethanol caused the usual concentration-dependent increases in firing rate. Quinidine (20 μM) significantly decreased the ethanol-induced excitation (two-way ANOVA, p < 0.001). B, Mean responses of 11 DA VTA neurons tested with ethanol before and in presence of 40 μM quinidine. Prior to quinidine, ethanol caused a concentration-dependent increase in firing rate. Quinidine (40 μM) significantly reduced the ethanol-induced excitation (two-way ANOVA, p < 0.001).
Fig. 7. Concentration-response curve for the quinidine reduction of ethanol-induced excitation. The mean percent change in firing rate of DA VTA neurons produced by 80 mM ethanol is plotted as a function of the concentration of quinidine present in the superfusate. The control point (0 μM quinidine) is the mean response of 21 DA VTA neurons, each of which was also tested with one or more concentrations of quinidine. Quinidine concentrations (1, 5, 10, 20 and 40 μM) were tested in 7, 8, 10, 9 and 5 neurons, respectively. Error bars indicate the S.E.M. Quinidine reduced the excitation produced by 80 mM ethanol in a concentration-dependent manner, with higher concentrations of quinidine causing larger reductions in the ethanol response. Responses to 80 mM ethanol in 10, 20 and 40 μM quinidine were significantly different from control (one-way ANOVA, p < 0.001, Student-Newman-Keuls post hoc comparisons, p < 0.005 in all cases).
Fig. 8. Quinidine blockade of ethanol excitation is fully reversible with washout of quinidine. The change in firing rate produced by 80 mM ethanol (as a percentage of baseline firing rate) was measured in 5 DA VTA neurons before and during administration of 40 μM quinidine and again 45-60 min after washout of quinidine. Ethanol caused similar increases in the firing rate of these neurons before quinidine (mean increase = 22.9 ± 3.2%) and after washout of quinidine (mean increase = 23.7 ± 1.7%). In the presence of 40 μM quinidine, the excitatory effect of ethanol was blocked and the mean change in firing rate in response to ethanol was a small decrease (-1.7 ± 4.2%). A one-way repeated measures ANOVA indicated significant differences among the treatment groups (p < 0.001) and Student-Newman-Keuls post-hoc comparisons indicated that the ethanol response in quinidine was significantly different from both the ethanol responses before quinidine and after washout of quinidine (both p < 0.001), and that ethanol responses before quinidine and after washout of quinidine did not differ (p > 0.05).
Figure 2

Change in firing rate (%) vs. Ethanol concentration (mM)

- Control
- ZD7288 30 μM
Figure 4

A

B

C

EtOH 80 mM  EtOH 40 mM  EtOH 120 mM  DA 5μM

Firing rate (Hz)

Time (min)

EtOH 80 mM  EtOH 40 mM  EtOH 120 mM  DA 5μM

Firing rate (Hz)

Time (min)

- Control
- 500 μM Barium

Increase in firing rate (%)

Ethanol concentration (mM)
Figure 5

A

Quinidine 40 μM
Quinidine 20 μM
Control

B

Quinidine 20 μM

Ethanol 80 mM
Ethanol 80 mM

Firing rate (Hz)

Time (min)
Figure 7

Change in firing rate (%) produced by 80 mM ethanol

Quinidine concentration (μM)