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Enantioselective Blockade of T-type Ca^{2+} Current in Adult Rat Sensory Neurons by a Steroid That Lacks γ-Aminobutyric Acid-Modulatory Activity

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ABSTRACT

A number of steroids seem to have anesthetic effects resulting primarily from their ability to potentiate currents gated by γ-aminobutyric $acid_A$ ($GABA_A$) receptor activation. One such compound is (3α,5α,17β)-3-hydroxyandrostane-17-carbonitrile [(+)-ACN]. We were interested in whether carbonitrile substitution at other ring positions might result in other pharmacological consequences. Here we examine effects of (3β,5α,17β)-17-hydroxyestrane-3-carbonitrile [(+)-ECN] on $GABA_A$ receptors and Ca^{2+} channels. In contrast to (+)-ACN, (+)-ECN does not potentiate $GABA_A$-receptor activated currents, nor does it directly gate $GABA_A$-receptor mediated currents. However, both steroids produce an enantioselective reduction of T-type current. (+)-ECN blocked T current with an IC_{50} value of 0.3 μM with a maximal block of 41%. (+)-ACN produced a partial block of T current (44% maximal block) with an IC_{50} value of 0.4 μM. Block of T current showed mild use- and voltage-dependence. The (−)-ECN enantiomer was about 33 times less potent than (+)-ECN, with an IC_{50} value of 10 μM and an amount of maximal block comparable to (+)-ECN. (+)-ECN was less effective at blocking high-voltage-activated Ca^{2+} current in DRG neurons (IC_{50} value of 9.3 μM with maximal block of about 27%) and hippocampal neurons. (+)-ECN (10 μM) had minimal effects on voltage-gated sodium and potassium currents in rat chromaffin cells. The results identify a steroid with no effects on $GABA_A$ receptors that produces a partial inhibition of T-type Ca^{2+} current with reasonably high affinity and selectivity. Further study of steroid actions on T currents may lead to even more selective and potent agents.

Because low-voltage-activated, or T-type, calcium (Ca^{2+}) currents are activated at potentials as negative as −60 mV, they are thought to play a key role in the initiation of regenerative depolarizing inward current (reviewed by Huguenard, 1996). The properties of T currents and their distribution in particular cell types suggest a critical role in the regulation of excitability in both neurons (Llinas, 1988; Huguenard and Prince, 1992) and other excitable cells (Matteson and Armstrong, 1984; Hirano *et al.*, 1989). T currents have also been proposed to contribute to initiation of seizure activity in thalamic neurons (Huguenard and Prince, 1994; Tsakiridou *et al.*, 1995). Thus, physiological regulation of T-type current is likely to be of profound significance to the regulation of neuronal activity.

In contrast to the abundance of peptide toxins that have proven useful in identifying the physiological roles of HVA variants of Ca^{2+} current (review by DeWaard *et al.*, 1996), there is an absence of highly potent and selective antagonists for T-type channels. Except for recent reports of the T-current blocking effects of mibefradil (Mishra and Hermsmeyer, 1994), a compound which also affects HVA types of Ca^{2+} current at somewhat higher concentrations (Bezprozvanny and Tsien, 1995), most other T current blockers are of relatively weak potency and selectivity. However, T-type currents are blocked at high concentrations by a variety of compounds within concentrations that are perhaps clinically relevant. This includes anesthetics (Herrington *et al.*, 1991; Study, 1994; Todorovic and Lingle, 1998) and also some anticonvulsants [e.g., succinimides (Coulter *et al.*, 1989a,

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ABBREVIATIONS: HVA, high-voltage-activated;(+)-ACN, (3α, 5α, 17β)-3-hydroxyandrostane-17-carbonitrile; (+)-ECN, (3β, 5α, 17β)-17-hydroxyestrane-3-carbonitrile; GVIA, ω-conotoxin GVIA; MVIIC, ω-conotoxin MVIIC; GDPβS, guanosine 5′-O-(2-thiodiphosphate); GTPγS, guanosine 5′-(γ-thio)triphosphate; $GABA_A$, γ-aminobutyric $acid_A$; DRG, dorsal root ganglion; R_s, series resistance; C_m, whole-cell capacitance; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulfoxide; HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid;

1989b; but see Leresche *et al.*, 1998) and phenytoin (Todorovic and Lingle, 1998)]. Selective blockers of T-currents are therefore likely to have interesting and important consequences on neuronal excitability and may be of unique clinical use.

The modulation of ion channel function by steroids is an area of increasing interest. Much of this interest has been focused on the modulation of $GABA_A$ receptor function by steroids (reviewed by Lambert *et al.*, 1995). With regard to Ca^{2+} channel modulation, some steroids have been reported to modulate Ca^{2+} currents through G-protein-mediated pathways (ffrench-Mullen *et al.*, 1994). Additionally, we have shown that (+)-ACN (Fig. 1), a steroid that powerfully potentiates and gates $GABA_A$ receptors (Wittmer *et al.*, 1996), exerts somewhat selective, direct blocking effects on particular components of HVA Ca^{2+} currents (Nakashima *et al.*, 1998). As part of ongoing structure-activity studies, we are examining other steroids for effects on Ca^{2+} currents. Here we describe the effects of (+)-ECN. (+)-ECN is a 5α-reduced steroid without a C-19 methyl group (a 19-norsteroid). Relative to (+)-ACN, the ring positions of the carbonitrile and hydroxyl groups are reversed. The stereochemical relationship between these two groups is also different.

The main finding of this study is that (+)-ECN (Fig. 1), which has no effect on GABA receptors at 10 μM, is a potent, enantioselective, partial blocker of T-type current in rat DRG sensory neurons. Furthermore, we compare the blocking effects of (+)-ECN to the action of two other steroids that exhibit anesthetic effects, (+)-ACN and alphaxalone. Although all three compounds share similarities in their effects on T type currents, (+)-ECN is unique in lacking any effect on $GABA_A$ receptors. Steroid analogues that exhibit relatively selective, potent, and reversible effects on T currents, without any effects on GABA receptors, may provide useful tools for examining the role of T currents in neuronal excitability and aid the potential development of compounds that may mediate anesthetic, analgesic, or anticonvulsant effects.

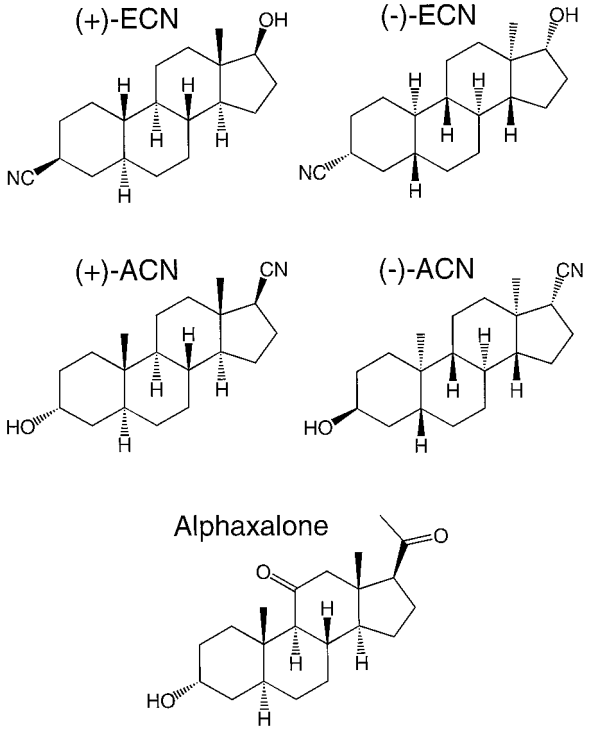

Fig. 1. Structures of (+)-ECN, (−)-ECN, (+)-ACN, (−)-ACN, and alphaxalone.

Materials and Methods

Preparation of cells. Acutely dissociated DRG neurons from adult male Sprague-Dawley rats (100–300 g) were obtained using enzymatic treatment as described elsewhere (Todorovic and Lingle, 1998). Glass coverslips with adherent DRG cells were transferred to a standard culture dish with a total volume <1 ml. Most results from DRG neurons were obtained from smaller diameter cells with no visible processes. Average uncompensated R_s was 6.6 ± 2.5 MΩ (mean ± standard deviation) and average C_m was 13.5 ± 4 pF for 217 neurons.

Microisland cultures of neonatal rat hippocampal neurons were prepared as described previously (Mennerick *et al.*, 1995). Cells used for recordings of HVA Ca^{2+} currents were used after 2–5 days in culture. Chromaffin cells were prepared from adult rat adrenal glands as described elsewhere (e.g., Solaro *et al.*, 1995).

Electrophysiological methods, solution application, and current isolation procedures. Currents were recorded using standard whole-cell patch-clamp methods (Hamill *et al.*, 1981). Solutions were applied to cells through multiple independently controlled glass capillary tubes, and solution was removed from the other end of the chamber with the use of constant suction. Solution application was accomplished by manually controlled valves. Test solutions were maintained in all-glass syringes and allowed to fall by gravity. Changes in Ca^{2+} current amplitude in response to rapidly acting drugs or ionic changes were typically complete in 10–20 sec. Switching between separate perfusion syringes, each containing control saline, resulted in no changes in Ca^{2+} current. For all steroids examined here, no dependence on the order of presentation or desensitization with repeated applications was observed.

The intracellular saline for recording of T current consisted of: 135–140 mM tetramethylammonium hydroxide, 10 mM EGTA, 40 mM HEPES, and 2 mM $MgCl_2$. The intracellular saline was usually titrated to pH 7.15–7.20 with HF, although in some experiments HCl or methanesulfonic acid was used. HVA currents were blocked by procedures described previously (Todorovic and Lingle, 1998). Specifically, experiments on T currents were done on smaller DRG neurons that express L- and N-type HVA current almost exclusively (Scroggs and Fox, 1992). Thus, in most experiments, the addition of F^- to the intracellular solution was used to abolish L-type HVA current as described previously (Herrington and Lingle, 1992; Todorovic and Lingle, 1998). In addition, such cells were preincubated with 1 μM GVIA to abolish N-type HVA current. For generation of concentration-response curves, T currents were elicited by voltage steps to −30 mV from a holding potential of −90 mV. This resulted in T current with minimal HVA current contamination (e.g., Todorovic and Lingle, 1998).

For recording of HVA currents, cells were held at −60 mV and inward currents were elicited by a test step to −10 mV. For HVA currents, the intracellular solution contained: 110 mM Cs-methane sulfonate, 14 mM phosphocreatine, 10 mM HEPES, 9 mM EGTA, 5 mM Mg-ATP, and 0.3 mM Tris-GTP, pH adjusted to 7.15–7.20 with CsOH (standard osmolarity: 300 mOsM). To verify that the composition of the intracellular solution did not influence the sensitivity of T currents to steroid action, in some experiments, the internal saline used for recording HVA currents was also used for recording of T current. In such cases, to isolate T current, HVA current was blocked by preincubation of cells with 1 μM GVIA, and by also including 2 μM MVIIC and 5 μM nifedipine in the external solution, to block N-, P-, Q- and L-types of HVA current, respectively. The blocking effects of steroids on T current were identical with all of the procedures used to isolate T current, regardless of whether the intracellular anion was F^-, methanesulfonic acid, or Cl^-.

The standard extracellular saline for recording of T-type Ca^{2+} currents contained: 152 mM tetraethylammonium-Cl, 10 mM HEPES, and 10 mM $BaCl_2$, adjusted to pH 7.4 with tetraethylammonium-OH, osmolarity 316 mOsM. For recording of HVA Ca^{2+} currents in DRG neurons, a 5 mM Ba^{2+} solution was used. Recordings of HVA Ba^{2+} current in cultured hippocampal neurons followed procedures described previously (Nakashima *et al.*, 1998).

Recordings of GABA currents on cultured hippocampal neurons were done as described previously (Mennerick *et al.*, 1995; Wittmer *et al.*, 1996). The extracellular recording solution contained: 140 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.3. Recording pipettes were filled with a solution containing: 140 mM CsCl, 4 mM NaCl, 5 mM EGTA, 0.5 mM $CaCl_2$, 4 mM $MgCl_2$, and 10 mM HEPES, pH 7.3. In studies examining autaptic currents, CsCl was replaced by KCl and $MgCl_2$ was replaced with 2 mM Mg-ATP and 0.5 mM Na-GTP in the intracellular solution. GABA and steroids were applied for 500 msec using a pressure (20 p.s.i. air) ejection drug delivery system with a patch pipette positioned approximately 5 μm from the neuron. The concentrations reported here are those in the pipette and are an upper limit for the concentrations reaching the cell. Autaptic responses were evoked from a holding potential of −70 mV using 1.5-msec voltage steps to +20 mV applied every 30 sec.

To record Na^+ and K^+ currents from chromaffin cells, the external saline contained: 140 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 1.8 mM $CaCl_2$ and 2.0 mM $MgCl_2$ titrated to pH 7.4 with *N*-methylglucamine. For recording of Na^+ currents, the internal saline was identical to the one for HVA Ca^{2+} currents used for DRG neurons. The internal saline for recording of K^+ currents contained: 140 mM KCl, 20 mM KOH, 10 mM HEPES (H^+), 5 mM HEDTA with added $CaCl_2$ to make 10 μM $[Ca^{2+}]_i$ as defined by the EGTAEC program (E. McCleskey, Vollum Institute, Portland, OR).

Analysis of steroid effects on current amplitude and properties. The percent reduction in peak inward current carried by Ba^{2+} ions at a given steroid concentration was used to generate concentration-response curves. For each of these curves, all points are averages of multiple determinations obtained from at least five different cells. Only cells where at least two different concentrations of the same steroid were tested were used to construct concentration-response curves. Because the level of maximal block by different steroids was somewhat variable from cell to cell, only those cells in which a drug concentration producing a near maximal effect was tested were taken for analysis. On all concentration-response curves, vertical bars indicate standard errors. Mean values on all concentration-response curves were fit to the following function:

$$PB([\text{Steroid}]) = PB_{max}/(1 + (IC_{50}/[\text{Steroid}])^n) \quad (1)$$

where PB_{max} is the maximal percent block of peak T current, IC_{50} is the concentration that produces 50% of maximal inhibition, and n is the apparent Hill coefficient for blockade. Fitted values are reported with 95% linear confidence limits. The voltage-dependence of steady state inactivation was described with Boltzmann distribution:

$$I(V) = I_{max}/(1 + \exp[-(V - V_{0.5})/k]) \quad (2)$$

where I_{max} represents maximal activatable current, $V_{0.5}$ represents the voltage where half of the current is inactivated, and k (units of millivolts) represents the voltage dependence of the distribution.

Effects of (+)-ECN on current activation and inactivation time constants (τ_m and τ_h, respectively) were determined from the fit of the following form of a Hodgkin and Huxley (1952) current activation equation:

$$I(t) = A * [1 - \exp(-t/\tau_m)]^n * \exp(-t/\tau_h) \quad (3)$$

where $I(t)$ is the current (I) as a function of time (t), A is the maximal activatable current, τ_m is the activation time constant, τ_h is the inactivation time constant, and n is a term for the sigmoidicity in the activation process. For T current at potentials more positive than −20 mV, n was typically constrained to 1.0. In the absence of such constraint, for currents between −60 and 0 mV, n varied from about 1.8 to about 1.0.

Drugs and chemicals. The synthesis of (+)-ECN and (−)-ECN will be described elsewhere. The compounds had spectroscopic data (IR, NMR) consistent with the assigned structures and were shown to have the correct elemental composition by combustion analysis for C, H, and N. The preparation of (+)-ACN (Hu *et al.*, 1993) and (−)-ACN (Hu *et al.*, 1997) have been described previously. Alphaxalone was obtained from Sigma (St. Louis, MO). All steroids were dissolved in DMSO to make 10–30 mM stock solutions. Aliquots of the stock solutions were added to the standard external solution to make the final concentrations given in the text. The final concentration of DMSO was less than 0.6% in these experiments; this concentration of DMSO did not affect I_{Ba} (data not shown, n = 5 cells) or $GABA_A$ currents. At steroid concentrations of 60 μM or higher, the steroids seem to crystallize out of solution, thus reducing the effective steroid concentration in solution. This has prevented us from using higher concentrations of steroids in experiments where maximal blockade could not be determined reliably.

GVIA (RBI, Natick, MA; Sigma) and MVIIC (RBI; Sigma) were dissolved in distilled water to make stock solutions of 0.5, 0.5 and 0.2 mM, respectively. Nifedipine (RBI) was dissolved in DMSO at 5 mM as a stock solution. GDPβS and GTPγS were obtained from Sigma and, when used, replaced GTP in the pipette solution.

Results

(+)-ECN does not potentiate GABA currents in rat hippocampal neurons. Fig. 1 displays the structures of the various steroids used in this investigation. A feature of many neuroactive steroids is their ability to potentiate $GABA_A$-receptor mediated Cl^- currents (Lambert *et al.*, 1995; Wittmer *et al.*, 1996). Both (+)-ACN (Wittmer *et al.*, 1996) and alphaxalone (Sear, 1996) are anesthetic steroids whose effects are thought to be mediated by $GABA_A$-receptor potentiation.

The effects of (+)-ECN on $GABA_A$-receptor-mediated currents were examined in cultured neonatal hippocampal neurons grown in microisland cultures (Fig. 2A). (+)-ECN (10 μM) was totally without effect on currents activated by 2 μM GABA. For comparison, 10 μM (+)-ACN produces a large potentiation of currents activated by 2 μM GABA (Fig. 2B; Wittmer *et al.*, 1996). (−)-ECN does not produce any significant potentiation of $GABA_A$-mediated currents at 10 μM (not shown), whereas (−)-ACN produces some potentiation (Wittmer *et al.*, 1996).

To ascertain other potential targets of (+)-ECN action, we also examined the effects of 10 μM (+)-ECN on autaptically evoked synaptic currents in the hippocampal microisland cultures (Mennerick *et al.*, 1995). (+)-ECN (10 μM) had no effect on either GABA-mediated inhibitory synaptic currents (Fig. 2C; two experiments) or glutamate-mediated excitatory synaptic currents (Fig. 2D; four experiments).

(+)-ECN inhibits T-type Ca^{2+} current in rat DRG neurons. T-type Ca^{2+} currents were isolated as described in Materials and Methods and typically monitored with voltage-steps to −30 mV from a holding potential of −90 mV. (+)-ECN reversibly depressed the amplitude of T current as seen in Fig. 3A without apparent effects on current activation or inactivation kinetics. Blockade by (+)-ECN was concentration-dependent (Fig. 3B) from 0.1 to 10 μM. In most cells, blockade by 30 μM (+)-ECN was indistinguishable from

blockade produced by 10 μM (+)-ECN. The percent block of peak T current by 10 μM (+)-ECN was 41.6 ± 10.7% for 36 cells. The blocking effect was strongly enantioselective, as shown in Fig. 3C, where 10 μM (+)-ECN blocked about 2-fold more T-type current than the same concentration of (−)-ECN. No apparent desensitization was obvious when cells were exposed sequentially to the same concentration of steroid. From such experiments, concentration-response curves for both agents were generated as depicted in Fig. 3D. In each cell, responses to any application of steroid were normalized to blockade produced by 10 μM (+)-ECN. For (+)-ECN, the IC_{50} for blockade of T-type Ca^{2+} current was 0.3 ± 0.02 μM with a Hill coefficient of 0.98 ± 0.07 (n = 15 cells). (−)-ECN was about 30-fold less potent with an IC_{50} value of 10 ± 1.6 μM and a Hill coefficient of 1.2 ± 0.2 (n = 10 cells). At concentrations producing maximal block, both compounds were about equally efficacious.

The partial blockade of T current by (+)-ECN raises the question of whether the blocking effect of (+)-ECN is directly on the T channel or via some modulatory pathway regulating T-current behavior. Four observations suggest that (+)-ECN does, in fact, block T current directly. First, T current inhibition by a given concentration of (+)-ECN is readily reversible and reproducible over sequential applications. Inhibition of Ca^{2+} currents by G-protein mediated pathways often exhibits a characteristic desensitization (Ikeda and Schofield, 1989; Shapiro and Hille, 1993). Second, the blocking effect of (+)-ECN was observed with an intracellular saline that lacked either ATP or GTP, two constituents deemed necessary for maintenance of second-messenger mediated signaling pathways. Third, the addition of either 100 μM GTPγS or 2 mM GDPβS to the pipette saline did not alter the ability of (+)-ECN to inhibit T current. With GTPγS, 10 μM (+)-ECN blocked 34.5 ± 3.2% (four experiments) of the T current. With GDPβS, 10 μM ECN inhibited 36.3 ± 3.2% (three experiments) of the T current. Neither GTPγS or GDPβS resulted in appreciable run-down or run-up of T current. Finally, the presence or absence of F^- in the intracellular saline, an anion which stimulates many G-proteins, did not influence the blocking actions of (+)-ECN. However, we did observe some variability among cells in the maximal blocking effect of (+)-ECN on T current. This might occur if T current were partially contaminated by inactivating, (+)-ECN-resistant HVA current. Alternatively, the blocking mechanism may involve state-dependent features, perhaps influenced by modulatory pathways, which may exhibit cell-to-cell variability.

Blockade by (+)-ECN produces little change in T current kinetic behavior but exhibits mild voltage- and use-dependence. Many compounds are thought to inhibit ion channels either by plugging the ion permeation or by producing allosteric changes in channel gating, such that inactivated or closed states are favored. Such effects are often revealed by kinetic alterations in the channel gating behav-

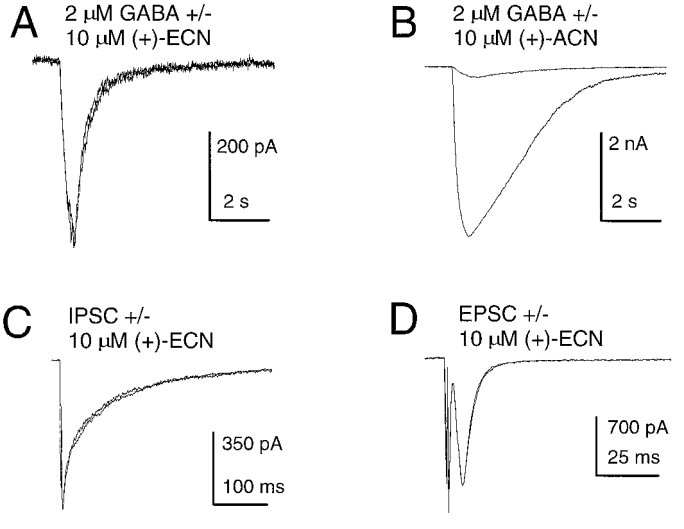

Fig. 2. A, *Traces* show currents activated by application of 2 μM GABA to a hippocampal neuron either with or without 10 μM (+)-ECN. B, *Traces* show currents activated by 2 μM GABA, but in the presence and absence of 10 μM (+)-ACN. Neurons shown in A and B were held at −60 mV. C, Stimulation of a hippocampal neuron grown in microisland culture resulted in an inhibitory autaptic current. (+)-ECN (10 μM) had no effect on the amplitude of the evoked inhibitory current. D, Stimulation of a hippocampal neuron resulted in an excitatory autaptic current. (+)-ECN (10 μM) had no effect. In C and D, stimulus artifacts have been truncated for clarity.

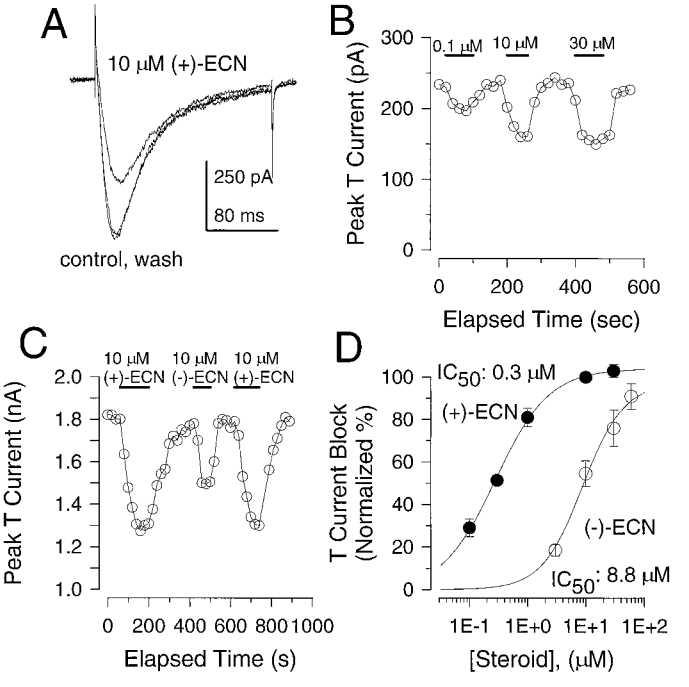

Fig. 3. (+)-ECN is a potent, enantioselective antagonist of T-type Ca^{2+} current in rat DRG cells. A, *Traces* show inward T-type Ca^{2+} currents activated from a holding potential of −90 mV to a test potential of −30 mV before, during and after application of 10 μM (+)-ECN. Note that current activation and inactivation rates are not obviously altered by (+)-ECN, despite the ~37% reduction in current amplitude. All current amplitudes are measured from the peak current to the current amplitude at the end of test pulse. C_m, 20 pF; R_s, 10 MΩ. B, The peak T current amplitude is plotted over the course of an experiment in which three different concentrations of (+)-ECN were applied to a DRG neuron (C_m, 17 pF; R_s, 11 MΩ). *Horizontal bars*, times of steroid application. Note that 30 μM steroid did not depress peak current amplitude more than that by 10 μM. C, Block of T current by identical concentrations of the (+) and (−) enantiomers of ECN is compared. At these concentrations, (−)-ECN was about half as effective. The similarity in response to both applications of 10 μM (+)-ECN indicates that desensitization between applications does not occur (C_m, 21 pF; R_s, 5 MΩ). D, Concentration-response curves to (+)- and (−)-ECN are plotted. *Points* (*open symbols*, (−)-ECN; ●, (+)-ECN) are averages of at least five different cells and are normalized to effect of 10 μM (+)-ECN within the same cell. *Solid line*, best fit from eq. 1 (see Materials and Methods); *vertical lines*, mean ± standard error. For (+)-ECN, the IC_{50} value was 0.3 ± 0.02 μM with a Hill coefficient of 0.98 ± 0.07 (15 cells), whereas for (−)-ECN, the IC_{50} value was 10 ± 1.6 μM with a Hill coefficient of 1.2 ± 0.2 (10 cells).

ior. To provide initial clues concerning possible mechanisms of (+)-ECN action, we next examined the effects of (+)-ECN on several aspects of T-current behavior.

Effects of (+)-ECN on T-current deactivation were examined at potentials from −160 through −60 mV following a 15-msec depolarizing step to −30 mV. Tail currents were reasonably well described by single exponential functions over this range and 10 μM (+)-ECN had no obvious effect on current deactivation (Fig. 4A). Current activation time constants were determined from fits of a Hodgkin-Huxley model (eq. 3) to T currents activated during a 380-msec depolarizing step to potentials between −65 mV and +30 mV. Values for n ranged from 2.0 to near 1.0, being near 1.0 at potentials of −20 mV and more positive. Thus, the Hodgkin-Huxley term τ_m approximates a single exponential fit to the rising phase of the current. (+)-ECN (10 μM) had no obvious effect on the rates of T current activation.

Potential effects of (+)-ECN on the rate of current inactivation were also determined from the value of τ_h in the fit of eq. 3 to the current waveforms. Values plotted in Fig. 4B indicate that 10 μM (+)-ECN had no significant effect on the time constant of current inactivation.

Recovery from inactivation was examined with a paired-pulse protocol in which a 100-msec step to −30 mV was first used to inactivate most T current. After a variable recovery interval (25 to 10,000 msec) at either −90 mV or −130 mV, a second test step to −30 mV was used to determine the amount of T current that had recovered from inactivation during the recovery period. The percent recovery in the presence and absence of 10 μM (+)-ECN for four cells was then plotted as a function of recovery duration at either −90 mV (Fig. 4C) or −130 mV (Fig. 4D). Recovery time courses with and without (+)-ECN were best fit with two exponential components with values given in the legend of Fig. 4. The time constants of recovery are similar both with and without (+)-ECN. However, the relative amplitude of the fast recovery component is somewhat smaller in (+)-ECN, resulting in a somewhat slower overall recovery.

We next determined whether (+)-ECN might alter T current availability at different conditioning potentials. T currents were evoked by a voltage-step to −30 mV after a 5-sec conditioning step at potentials from −110 to −55 mV in the presence and absence of 10 μM (+)-ECN (Fig. 5A). This procedure defines the voltage-dependence of T-current fractional availability (Todorovic and Lingle, 1998). Fig. 5A shows that 10 μM (+)-ECN reduced T current elicited from negative potentials by almost 50%. The normalized maximal current elicited from each conditioning potential is plotted as a function of the conditioning potential in Fig. 5B for a set of eight cells. Fig. 5B, *solid lines*, represent the best fits from the Boltzmann equation (eq. 2); for control conditions, half availability occurred at −78 mV with slope factor of 8 mV, whereas in the presence of 10 μM (+)-ECN, the $V_{0.5}$ was −85.5 mV with a slope factor of 8.8 mV. These experiments indicate that (+)-ECN exerts a somewhat stronger blocking effect at more positive conditioning potentials, but the effect is rather small. The slight slowing of recovery from inactivation observed in Fig. 4, C and D, might contribute to the effect of (+)-ECN on steady state inactivation.

The dependence of the fractional block of T current by (+)-ECN on T current stimulation frequency was also examined. In control conditions, when T currents are activated by 250-msec depolarizations applied every 20 or 5 sec, no change in peak T current amplitude is noted (Fig. 5C). However, in the presence of 10 μM (+)-ECN, activation of T current at 1 per 5 sec increases the amount of blockade by (+)-ECN by about 25% relative to blockade at 1 per 20 sec. The average increase in block (when cells are stimulated every 20 versus every 5 sec) was 25 ± 8% (mean ± standard deviation) (n = 7 cells) for 10 μM (+)-ECN. This result is consistent with the somewhat stronger blockade of T current by (+)-ECN at more positive potentials. At higher stimulation frequencies, the recovery time at −90 mV is insufficient to allow full recovery from the blockade developed at −10 mV.

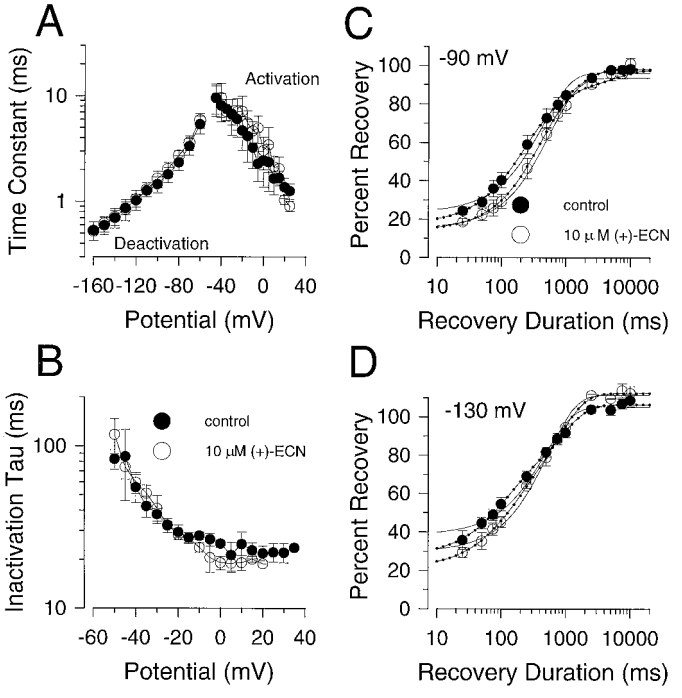

Fig. 4. Effects of (+)-ECN on kinetic properties of T currents in DRG neurons. A, Tail currents were examined at potentials from −160 mV to −60 mV after current activation at −30 mV. Deactivation values are the mean decay time constants for 4 cells (mean ± standard deviation). Activation values are mean ± standard deviation from four cells of τ_m values from eq. 3 (Hill coefficient ~ 1.0 to 1.4). *Solid symbols*, control saline; ○, 10 μM (+)-ECN. B, Symbols plot mean values for four cells of t_h values from eq. 3. C, Percent recovery of peak inactivating current is plotted as a function of recovery duration at −90 mV. *Symbols*, mean ± standard deviation for four cells. Solid line is single exponential fit, whereas dotted line is two exponential fit. Non-zero initial recovery reflects incomplete T current inactivation during initial 100-msec step to −30 mV and/or some unblocked, noninactivating HVA current contamination of the peak current amplitude. For single exponential fits, recovery time constants were 448.9 ± 59.1 msec (control) and 619.5 ± 61.8 msec [10 μM (+)-ECN]. From the fit of a two exponential function, in control saline the fast time constant (τ_f) was 186 ± 25.2 msec (59.1%) with a slow time constant (τ_s) of 1171 ± 232.8 msec. In 10 μM (+)-ECN, τ_f was 407.9 + 129 msec (72.5%) and τ_s was 2123 ± 1450 msec. However, in 10 μM (+)-ECN, a reasonable fit could also be obtained by constraining τ_f and τ_s to the values obtained in control saline with the relative amplitude of τ_f reduced to 45.8%. D, Fractional recovery at −130 mV with and without 10 μM (+)-ECN is plotted. Single exponential time constants were 489.3 ± 82.6 msec and 556.5 ± 59.3 msec, for control and 10 μM (+)-ECN, respectively. For two exponential components, in control saline τ_f was 95.8 ± 67.2 msec (41.8%) and τ_s was 830.8 + 227 msec, whereas in 10 μM (+)-ECN τ_f was 103.5 ± 59.5 msec (29.0%) and τ_s was 760.2 ± 128.6 msec.

(+)-ECN is relatively ineffective at blocking HVA current in rat DRG neurons. A number of steroids have been reported to inhibit HVA types of Ca^{2+} currents (ffrench-

Mullen and Spence, 1991; Spence *et al.*, 1991; ffrench-Mullen *et al.*, 1994). Recently, we have shown that (+)-ACN, another neuroactive steroid, blocks N-, Q-, and R-type HVA currents but not L- or P-type currents in DRG and hippocampal neurons, with IC_{50} values in the range of 5–20 μM (Nakashima *et al.*, 1998). To examine the effectiveness of (+)-ECN on HVA current, cells were held at −60 mV and largely noninactivating currents were evoked by depolarizing steps to −10 mV. HVA current in these cells was composed primarily of nifedipine-sensitive L-type current and GVIA-sensitive N-type current (Scroggs and Fox, 1992; Todorovic and Lingle, 1998). In Fig. 6A, a cell that exhibited both T-type and HVA current is depicted. T-type current was initially evoked by a test step to −40 mV from a holding potential of −90 mV; after return to a holding potential of −50 mV, a step to 0 mV resulted in activation of a largely noninactivating HVA current. (+)-ECN (1 μM) produced a partial inhibition of the peak T current but had no effect on current activated by the subsequent step to 0 mV. Fig. 6B illustrates traces of HVA currents from the same cell before, during, and after application of 30 μM (+)-ECN, which produced a reversible, 22% reduction of HVA current. Fig. 6C illustrates the time course of HVA current blockade in another rat DRG cell. (+)-ECN (3, 30, and 60 μM) reversibly reduced the peak HVA current amplitude in a concentration-dependent manner with a maximal block of about 27%. In this cell, 1 μM GVIA irreversibly blocked about 16% of the total HVA current indicative of N-type current blockade, whereas 5 μM Nifedipine (an "L" type antagonist) blocked most of the remaining HVA current in this cell. For this cell, this result indicates that at least most of the current blocked by 60 μM (+)-ECN is primarily L-type current. Fig. 5D displays the concentration-response curve for blockade of total HVA current by (+)-ECN in rat DRG cells. All points are an average of at least five cells (total $n = 11$ cells). In Fig. 5D, the *solid line* is a best fit of eq. 1, yielding an IC_{50} value of 9.3 ± 2.7 μM, with a Hill coefficient of 1.2 ± 0.3 and a fitted maximal block of 27.6 ± 3%.

In other experiments, the effect of (+)-ECN was examined on isolated N- or L-type HVA current. For these experiments, small DRG neurons, which express predominantly N- and L-type HVA currents (Scroggs and Fox, 1992), were used. For five DRG neurons in which N-type current was abolished with 1 μM GVIA, (+)-ECN blocked a maximum of 37 ± 8% of the residual, predominantly L-type current with an IC_{50}

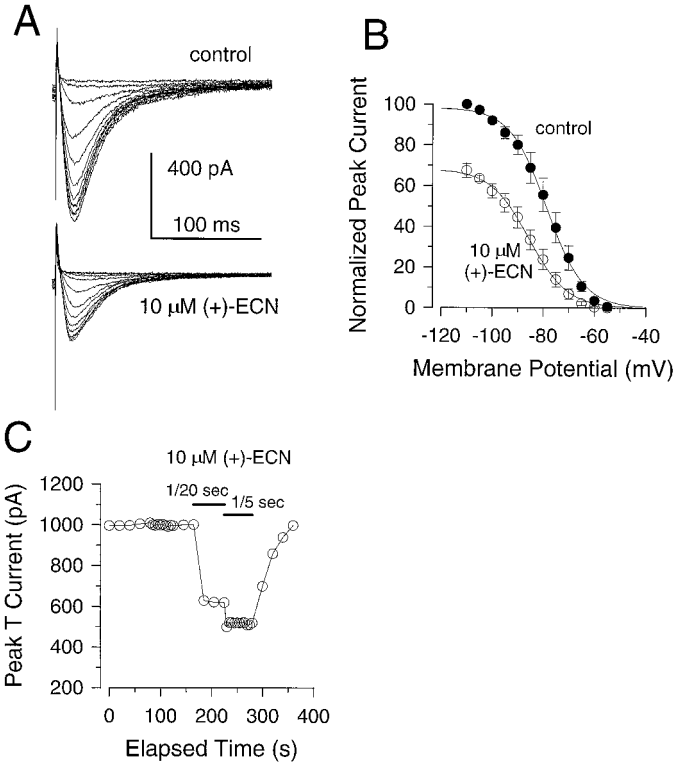

Fig. 5. Availability of T current for activation is influenced by (+)-ECN. A, *Traces* show currents activated by voltage steps to −10 mV after a 5-sec step to potentials from −110 through −55 mV either in control saline (*top*) or 10 μM (+)-ECN (*bottom*). C_m, 16 pF; R_s, 10 MΩ. B, The average fractional availability of T current as a function of voltage is plotted for control and 10 μM (+)-ECN for eight cells. *Error bars*, mean ± standard error; *solid lines*, best fit of eq. 1. For control saline, half inactivation occurred at −78 mV with a slope factor of 8 mV; in the presence of 10 μM (+)-ECN, the $V_{0.5}$ was −85.5 mV with a slope factor of 8.8. C, T currents were elicited once every 5 sec or once every 20 sec, in the absence and presence of 10 μM (+)-ECN. The change in stimulus frequency has no effect on T current amplitude under control conditions, but in the presence of (+)-ECN, peak T current amplitude is reduced at higher stimulus frequencies (C_m, 13 pF; R_s, 12 MΩ).

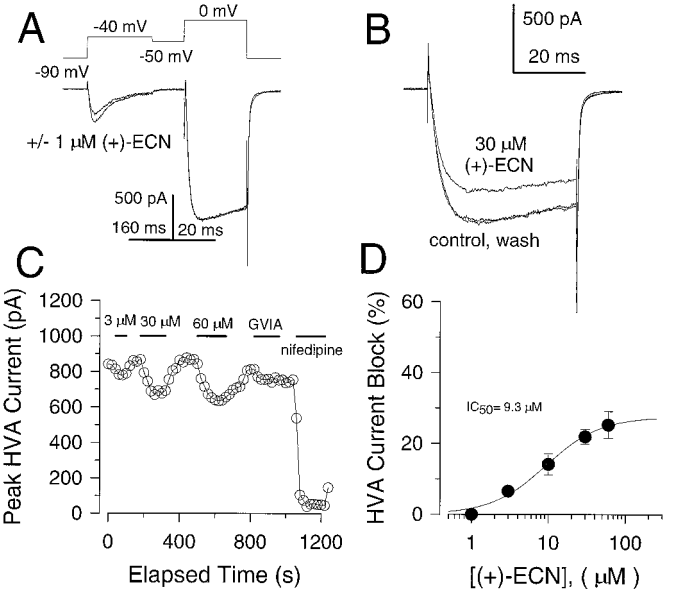

Fig. 6. Effects of (+)-ECN on HVA currents in rat DRG neurons. A, *Traces* show currents in the absence and presence of 1 μM (+)-ECN. Currents were activated with the voltage protocol shown on the top. A step to −40 mV was used to activate and inactivate T current; after repolarization to −50 mV, a step to 0 mV was used to activate HVA current. (+)-ECN (1 μM) blocks about 20% of the inactivating current at −30 mV but has no effect on current activated at 0 mV. Note the different time bases used for acquisition of LVA and HVA currents. *Vertical calibration bar*, time at which the sampling interval was changed from 0.8 msec to 0.1 msec. B, *Traces* show currents activated from a voltage step to −10 mV from a holding potential of −60 mV before, during, and after application of 30 μM (+)-ECN from the same cell used in A. About 22% of the total HVA current was blocked. C_m, 10 pF; R_s, 5 MΩ. In C, a time record of peak HVA current amplitude from another DRG cell shows the relative blocking effect of 3, 30, and 60 μM (+)-ECN. *Horizontal bars*, times of steroid application. Note that 60 μM steroid blocked only slightly more current than 30 μM. Comparing the effect of GVIA and nifedipine indicates that most HVA current in this cell was of L-type. C_m, 17 pF; R_s, 9 MΩ. D, The concentration-dependence of blockade of total HVA current by (+)-ECN is displayed. Smaller size DRG cells containing primarily N- and L-type Ca^{2+} currents were used (Scroggs and Fox, 1992). *Points*, averages of at least five different cells; *vertical lines*, mean ± standard error. *Solid line*, best fit of eq. 1, yielding an IC_{50} value of 9.3 ± 2.7 μM, a Hill coefficient of 1.2 ± 0.3, and maximal block of 27.6 ± 3% (8 cells).

value of 12.7 ± 6.6 μM (n = 1.6 ± 0.8). For five DRG neurons in which L-type current was abolished by a combination of intracellular F^- and application of 5 μM nifedipine, (+)-ECN blocked a maximum of 44 ± 4.6% of the residual, predominantly N-type current with an IC_{50} value of 8.7 ± 2.1 μM (n = 1.5 ± 0.4). Thus, both N- and L-type current are only weakly sensitive to (+)-ECN.

We also examined the effects of (+)-ECN on total HVA current in cultured neonatal rat hippocampal neurons. In such neurons, HVA current is typically composed of at least 5 distinguishable components. (+)-ACN has previously been shown to block N-, Q-, and R-types of HVA current in these cells, with IC_{50} values of 10–25 μM, but does not affect L- and P-type current (Nakashima *et al.*, 1998). Here, we simply compared the block produced by 30 μM (+)-ECN with that produced by 30 μM (+)-ACN. 30 μM (+)-ECN blocked an average of 21.1 ± 3.5% of total HVA current (n = 5) (data not shown), whereas effects of 10 μM (+)-ECN on HVA current were difficult to discern. Blockade by 30 μM (+)-ECN was 50.2 ± 4.5% of the blockade produced by 30 μM (+)-ACN. Thus, in sum, (+)-ECN seems to produce partial blocking effects on some HVA current components, but with relatively weak effects at less than 10 μM.

The effects of (+)-ECN on voltage-gated Na^+ and K^+ currents in rat chromaffin cells. We next examined the effects of (+)-ECN on several other potential ion channel targets found in cultured adult rat adrenal chromaffin cells. Rat chromaffin cells express a robust, tetrodotoxin-sensitive voltage-dependent Na^+ current. Fig. 7A shows voltage-dependent Na^+ current before and during application of escalating concentrations of (+)-ECN. (+)-ECN (30 μM but not 1 and 10 μM) produces a slight reduction (~14%) in Na^+ current amplitude. Fig. 7B plots the time course of Na^+ current amplitude from the same experiment.

Rat chromaffin cells also express a robust BK-type Ca^{2+}- and voltage-dependent K^+ current which exhibits inactivation (Solaro *et al.*, 1995). This current can be observed in relative isolation by voltage steps to +90 mV when the recording pipette contains 10 μM Ca^{2+} (Fig. 7C). After inactivation of the BK current at +90 mV, there is also a persistent voltage-dependent K^+ current. Neither 10 nor 30 μM (+)-ECN had any effect on either the inactivating or sustained component of K^+ current. The lack of effect of (+)-ECN on either K^+ current is also shown in Fig. 7D.

From the above experiments, we conclude that at 10 μM (+)-ECN, a concentration maximally effective at blocking T current in rat DRG cells, there is no effect upon several other voltage-gated currents. Even at 30 μM (+)-ECN, effects on K^+ currents are nonexistent with only minimal effects on Na^+ current.

The effects of alphaxalone and (+)-ACN on T current in rat DRG cells. The enantioselectivity in the blocking effect of (+)-ECN on T-type Ca^{2+} current indicates that particular structural requirements are necessary for the blocking effect. Although a more thorough examination of the structural requirements of T-current block will be required, here we have examined the ability of three other steroids, alphaxalone, (+)-ACN, and (−)-ACN, to block T-type calcium current in rat DRG cells.

Alphaxalone is the only steroid anesthetic that has been widely used in human medicine (Sear, 1996). Fig. 8A shows traces of T current before, during, and after application of 30 μM alphaxalone, which in this cell blocked about 50% of peak T current. Alphaxalone, in contrast to (+)-ECN, is also a potent GABAergic agent (Lambert *et al.*, 1995). We were therefore concerned that the apparent reduction in outward current observed with alphaxalone might result from a $GABA_A$-receptor mediated activation of a superimposed outward current. To test this possibility, DRG neurons were stimulated with voltage ramps from −90 to 90 mV (data not shown) in the presence of cadmium to completely block inward current. Subsequent application of alphaxalone failed to evoke any inward or outward current, indicating that the apparent effects of alphaxalone on T-current do not arise from coincidental activation of a Cl^- current.

The effect of alphaxalone on the T-current steady state inactivation curves was also examined. As shown on Fig. 8B, the $V_{0.5}$ for T-current availability was shifted from −79.5 mV to −88.5 mV, with slope factors of 7.7 mV and 9.4 mV in the absence and presence of this steroid, respectively (n = 5 cells). The magnitude of this effect, although not profound, is comparable with the effect of (+)-ECN on DRG T current. Also similar to the effect of (+)-ECN, there was an increase in the fractional blockade by alphaxalone (26 ± 9%; n = 4 cells) as the frequency of stimulation of T current was increased

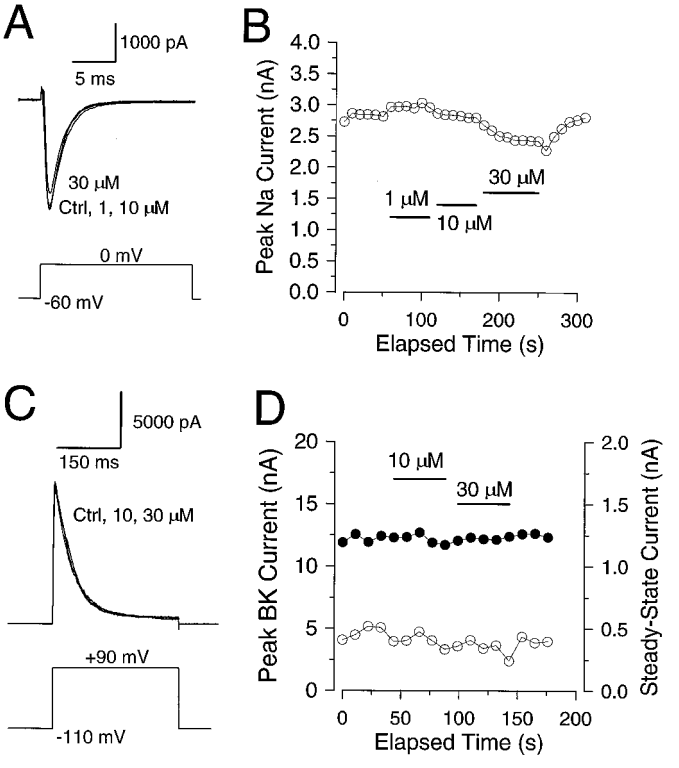

Fig. 7. Lack of effect of (+)-ECN on voltage-dependent Na^+ current (I_{Na}) and K^+ currents in rat adrenal chromaffin cells. A, *Traces* show I_{Na} activated by the indicated voltage protocol. *Traces* in 1 and 10 μM (+)-ECN are indistinguishable from control, whereas 30 μM produced a ~14% reduction in I_{Na}. B, Peak I_{Na} amplitude during the course of an experiment indicates that any effects of (+)-ECN are rather minor. C, *Traces* show outward currents evoked by steps to 90 mV from a holding potential of −110 mV with 10 μM pipette Ca^{2+}. The inactivating component of current is BK-type Ca^{2+}dependent K^+ current, whereas the residual noninactivating current reflects at least one other voltage-dependent K^+ conductance. Neither current component was affected by either 10 or 30 μM (+)-ECN. D, Peak current amplitude is plotted as a function of experimental time illustrating the lack of effect of (+)-ECN on both peak (●) and steady state (○) K^+ current components.

from every 20 to every 5 seconds (data not shown). Alphaxalone also shares with (+)-ECN a lack of any discernible effect on T current activation or inactivation kinetics (data not shown).

Blockade of T current by alphaxalone was concentration-dependent (Fig. 8C) with an IC_{50} value of 1.3 ± 0.3 μM, a Hill coefficient of 0.92 ± 0.14, and maximal block of 55 ± 12% (n = 15 cells). Alphaxalone at 10 μM had no effect on total HVA current in DRG cells (Fig. 8D; n = 2 cells) and had minimal effect upon total HVA current in hippocampal neurons (only 7% block of total HVA current at 30 μM; Nakashima *et al.*, 1998).

(+)-ACN is another steroid that, unlike (+)-ECN, has significant effects upon $GABA_A$ receptors (Fig. 2; Wittmer *et al.*, 1996). Potentiation of currents activated by 2 μM GABA occurs with an EC_{50} value of 1.4 μM (+)-ACN, whereas direct gating of $GABA_A$ receptor current by (+)-ACN occurs with an EC_{50} value of 5 μM (Wittmer *et al.*, 1996). In addition, (+)-ACN, in contrast to (+)-ECN and alphaxalone, exhibits blocking effects on specific subtypes of HVA Ca^{2+} currents in the range of 5–20 μM (Nakashima *et al.*, 1998).

In rat DRG cells, we found that (+)-ACN also produces enantioselective blockade of T currents. Maximal block was incomplete being about 40% and no increase in block was observed between 10 and 30 μM (+)-ACN (Fig. 9A). Effects of 10 μM (+)-ACN on steady state T current availability were similar to effects seen with (+)-ECN and alphaxalone (Fig. 9B). Blockade by (+)-ACN exhibited marked enantioselectivity (Fig. 9C). At 10 μM, (+)-ACN blocked 44 ± 13% of the T current (n = 36), with an IC_{50} value of 0.4 ± 0.07 μM and n of 1 ± 0.2. (−)ACN was about 50 times less potent with an IC_{50} value of 23.5 ± 11 μM, n of 1.4 ± 0.33 and a fitted amount of maximal T current blockade comparable with that produced by 10 μM (+)-ACN.

These results suggest that a number of steroids can block T current in DRG neurons with a high degree of enantioselectivity. Furthermore, the partial blockade of T current by these compounds, the lack of kinetic alterations by these compounds, and similar small shifts in steady state inactivation curves suggests that each of these compounds may block T current with a similar mechanism.

If different steroids were acting at different sites and by different mechanisms to produce blockade of T currents, some additivity in their blocking effects might be expected. To test this possibility, concentrations of (+)-ACN and (+)-ECN yielding near maximal blocking effects (10 μM in each case) were coapplied on the same cell (n = 7 cells; Fig. 9D) and compared with responses to a 10 μM concentration of each steroid alone. The amount of block when they were given together was not additive, which suggests that these

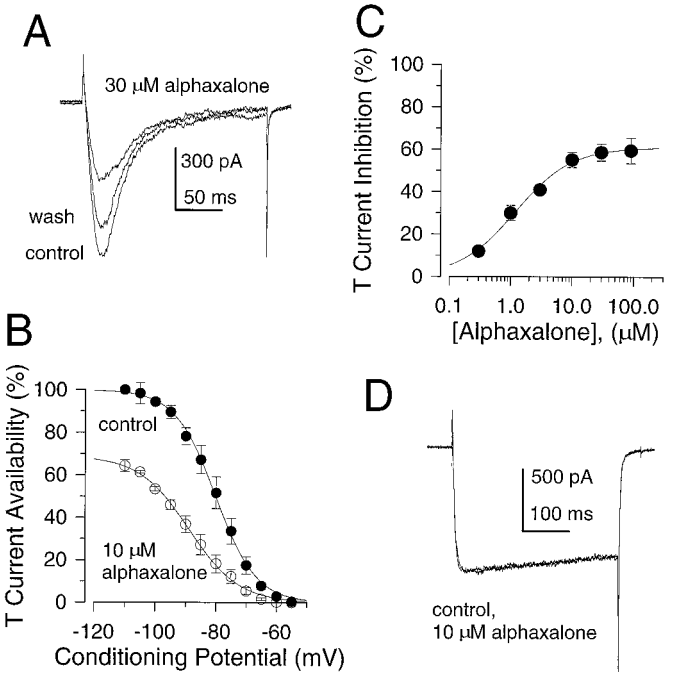

Fig. 8. Effects of alphaxalone on T currents in rat DRG cells. A, *Traces* show T current in a DRG cell before, during, and after application of 30 μM alphaxalone. B, The effect of alphaxalone on fractional availability of DRG T current is illustrated. *Solid lines*, fits of eq. 2. Alphaxalone shifted the $V_{0.5}$ from −79.5 mV (control, *open symbols*) to −88.5 mV (alphaxalone, *filled symbols*) with slope factors of 7.7 mV (control) and 9.4 mV (alphaxalone) (n = 5 cells). *Error bars*, mean ± standard error. C, Concentration-response curves for percent inhibition of T current by alphaxalone are plotted. *Points*, average of at least five different cells; *bars*, mean ± standard error. *Solid line*, best fit of eq. 1 with an IC_{50} value of 1.3 ± 0.3 μM, a hill coefficient of 0.92 ± 0.14, and a fitted maximal block of 62 ± 4.5% (n = 20 cells). D, HVA currents were activated by steps to −10 mV from −60 mV in the presence and absence of 10 μM alphaxalone. Alphaxalone had no effect.

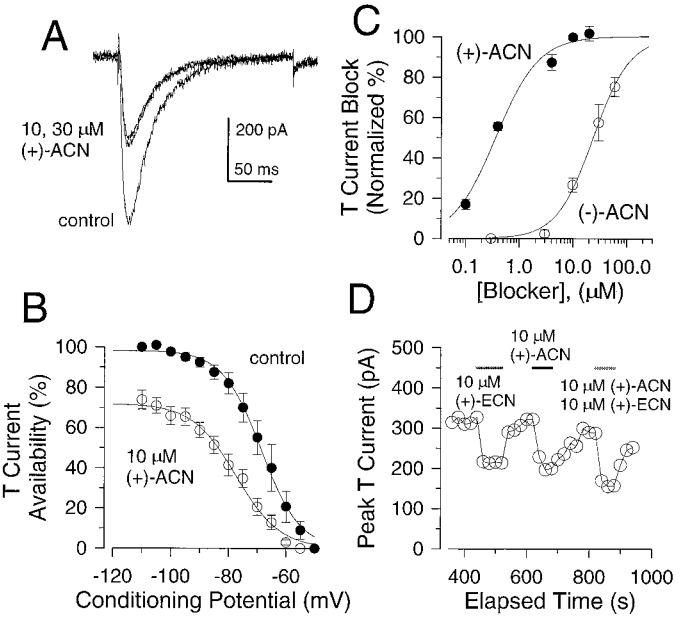

Fig. 9. Effects of (+)-ACN on T currents in rat DRG cells. A, *Traces* show inhibition of T current in a DRG cell by 10 and 30 μM (+)-ACN. Maximal block is achieved with concentrations of about 10 μM. B, The effect of 10 μM (+)-ACN on availability of DRG T current is plotted. *Solid lines*, fits of eq. 2. For control currents, the $V_{0.5}$ was −68.7 ± 0.7 mV with a slope factor of −6.7 ± 0.6 mV. In 10 μM (+)-ACN, the $V_{0.5}$ was −77.3 ± 1.1 mV with a slope factor of −7.6 ± 1.0 mV with a limiting maximal availability of 71.8 ± 2.7%. C, Concentration-response curves show inhibition of peak T current by (+)-ACN and its enantiomer, (−)-ACN. In each cell studied with (−)-ACN, responses were normalized to the block produced by 10 μM (+)-ACN obtained in the same cell. *Points*, averages of multiple determinations (at least five cells), *error bars*, mean ± standard error; *solid lines*, best fits of eq. 1. The IC_{50} value for block by (+)-ACN was 0.4 ± 0.2 μM with a Hill coefficient of 1.1 ± 0.6. For (−)-ACN, assuming a comparable maximal block, the IC_{50} value was 23.9 ± 2.4 μM with a Hill coefficient of 1.3 ± 0.2. D, Peak T current over the course of an experiment is plotted to illustrate the lack of additivity of the blocking effects of (+)-ACN and (+)-ECN. (+)-ACN (10 μM) and 10 μM (+)-ECN each produce a similar blocking effect, which is also comparable to block by the simultaneous application of 10 μM (+)-ACN and 10 μM (+)-ECN.

two steroids may act in a similar fashion, perhaps at the same site, to block neuronal T current.

Discussion

A novel neuroactive steroid, (+)-ECN, produces a potent blockade of T-type Ca^{2+} current in rat DRG neurons with 50% of the maximal blocking effect occurring at 0.3 μM. This effect is strongly enantioselective; (−)-ECN is more than 30 times less potent. Maximal blockade by (+)-ECN is only about 40% of total T current. Similarly, for all steroids studied here that do inhibit T current, maximal blockade was incomplete.

A number of other T current blockers have also been reported to produce an incomplete block at concentrations producing a maximal effect. For example, the anticonvulsants phenytoin and α-methyl-α-phenyl-succinimide also block less than 50% of DRG T current (Todorovic and Lingle, 1998). Partial block of other Ca^{2+} channel variants has also been described and, in the case of blockade of P-type current by ω-agatoxin IIIA, it has been proposed that a partial reduction of the rate of ion permeation through the P-type channel may account for the partial blocking effects (Mintz, 1994). In the case of T current block, the mechanism underlying the partial blockade produced by any compound remains unknown.

The anticonvulsant drug ethosuximide has been reported to block only about 40% of T current in thalamic neurons (Coulter *et al.*, 1989a, 1989b). However, recent work has failed to identify any effect of 0.5 mM ethosuximide on T current in thalamic neurons (Leresche *et al.*, 1998). In fact, other work indicates that T current can be maximally blocked by ethosuximide in both GH3 cells (Herrington and Lingle, 1992) and DRG neurons (Todorovic and Lingle, 1998), but only at concentrations (IC_{50} ~ 20–30 mM) that greatly exceed those used clinically.

Selectivity in blockade by (+)-ECN. In contrast to alphaxalone and (+)-ACN, (+)-ECN seems to be relatively selective in its ability to block T current and exerts little effect on other targets at comparable concentrations. Although maximal block of T current by (+)-ECN is only partial, this block is of relatively high affinity, producing half maximal block at about 0.3 μM. In contrast, at 10 μM, (+)-ECN has only small effects on HVA current in both rat DRG and hippocampal neurons. Providing additional support for the idea that (+)-ECN is relatively ineffective against HVA currents, we have observed that (+)-ECN has weak blocking effects on cloned human α1E Ca^{2+} channels expressed in HEK cells (Nakashima Y, Pereverzev A, Schneider T, Covey DF, and Lingle CJ. Blockade of Ba^{2+} current through human α1E channels by two steroid analogs, (+)-ACN, and (+)-ECN; submitted for publication.), blocking up to about 80% of the α1E current with an IC_{50} value of about 19 μM. Thus, HVA Ca^{2+} currents seem to be largely unaffected by (+)-ECN at concentrations (~1 μM) producing a near maximal effect on T currents. This apparently marked selectivity of (+)-ECN is also supported by the lack of effect on voltage-gated Na^+ and voltage dependent K^+ current and Ca^{2+}-dependent K^+ current in rat chromaffin cells. (+)-ECN therefore seems to exhibit a combination of potency and selectivity that may allow it to be of potential use in the pharmacological evaluation of T currents.

Blockade of T current by both (+)-ECN and (+)-ACN also exhibits strong enantioselectivity. This implies that the site affected by these steroids has quite specific structural requirements. Given the disparity in structure among (+)-ECN, (+)-ACN, and alphaxalone, is it possible that the blocking effects observed here represent effects on more than one target site? It is difficult to exclude this possibility. However, the fact that the blocking effects of (+)-ACN and (+)-ECN are not additive implies that, at least for these two structurally distinct steroids, there may be a common site and mechanism of action. Furthermore, several features of the block of T current produced by (+)-ECN, alphaxalone, and (+)-ACN support this view. In particular, all three compounds produce similar changes in steady state inactivation curves, each produces a partial block at maximal concentrations, and each has essentially no effect on kinetic properties of T currents. Although it is possible that each compound acting at distinct sites might result in this identical set of blocking characteristics, the simplest view at the present time is that they are acting at the same site.

Despite the similarity in action of (+)-ECN, (+)-ACN, and alphaxalone on T-type current, the lack of effect of (+)-ECN on GABA receptors seems particularly remarkable. There are multiple differences in the structures of (+)-ACN and (+)-ECN that might contribute to the selectivity of (+)-ECN in producing T channel inhibition, while leaving many other steroid-sensitive targets unaffected. These differences include: 1) the positions of the hydroxy and carbonitrile groups; 2) the relative stereochemistry between these groups; 3) the presence or absence of a C-19 methyl group; and 4) the distances between the oxygen and nitrogen atoms. Each of these differences needs to be evaluated more fully in future studies to understand its contribution to the ion channel selectivity observed in this study for (+)-ECN.

(+)-ECN, (+)-ACN, and alphaxalone also show interesting differences in their ability to inhibit HVA Ca^{2+} currents. Whereas both (+)-ECN and alphaxalone have relatively small effects on HVA Ca^{2+} currents, (+)-ACN seems to inhibit N-, Q-, and R-type currents with IC_{50} values in the range of 5–20 μM (Nakashima *et al.*, 1998). Thus, (+)-ECN and alphaxalone seem to share similar effects on T-type current and HVA currents, but differ in their effects on $GABA_A$ receptors. The lack of effect of (+)-ECN on HVA currents is also consistent with its lack of effect on inhibitory or excitatory synaptic currents.

Does T current inhibition result in interesting clinical/behavioral effects?. Until recently, T current inhibition has been the primary proposed explanation for the anticonvulsant actions of the succinimides (Macdonald and McLean, 1986; Coulter *et al.*, 1989a, 1989b). As noted above, this hypothesis has now been challenged by work that has failed to observe inhibition of T current by appropriate concentrations of ethosuximide (Leresche *et al.*, 1998). Yet, an important role of T current in convulsant activity is also suggested by the role of T current in burst generation in thalamic neurons (Huguenard and Prince, 1992) and the fact that increases in T current amplitude seem to favor epileptic discharges (Tsakiridou *et al.*, 1995).

At present, whether inhibition of T currents may contribute to other clinically or behaviorally important alterations remains unknown. However, the present results with alphaxalone may support this possibility. Alphaxalone remains the only anesthetic steroid that has been widely used in

human medicine. Interestingly, whereas T current blockade by alphaxalone occurs with an IC_{50} value of 1.3 μM, the reported values for alphaxalone in plasma during anesthesia in humans is in the range of 6.5–13 μM (Sear and Prys-Roberts, 1979). This suggests that, in mammals, alphaxalone affects T current in subanesthetic concentrations and, thus, T current inhibition is occurring during the production of anesthesia. On the other hand, it would seem unlikely that T current inhibition *per se* would contribute to the production of anesthesia.

It is interesting to consider several other aspects of the clinical action of alphaxalone in relation to a possible role of T current blockade. Alphaxalone has been reported to be a more efficacious agent in treatment of intractable *status epilepticus* than classic GABAergic agents like barbiturates (Chin *et al.*, 1979). It is also more effective in suppressing epileptic activity in experimental models than thiopental and diazepam (DeRiu *et al.*, 1987). In addition, alphaxalone has been reported to have stronger analgesic effects than propofol and pentobarbital (Gilron and Coderre, 1996). It is possible that these clinical effects of alphaxalone, which distinguish it from other general anesthetics, may result from effects on novel ion channel targets, perhaps T currents. Thus, T current inhibition by particular steroids may contribute both to anticonvulsant effects and analgesic consequences.

In conclusion, we have shown that several steroids inhibit T type Ca^{2+} currents at submicromolar concentrations. Furthermore, one of these compounds, (+)-ECN, produces these effects while exerting essentially no effects on $GABA_A$ receptors. The strong enantioselectivity in the blocking action of (+)-ECN indicates that T channels probably contain a steroid binding site with well-defined structural features. Over the range of concentrations effective on T current, (+)-ECN has essentially no effect on HVA Ca^{2+} currents, voltage-dependent Na^+ current, and some K^+ currents at concentrations affecting T currents. (+)-ECN and related compounds may prove useful in clarifying physiological and behavioral roles of T currents. Further work may lead to identification of compounds with even more potency and selectivity in blocking T currents.

Acknowledgments

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References

Bezprozvanny I and Tsien RW (1995) Voltage-dependent blockade of diverse types of voltage-gated channels expressed in *Xenopus* oocytes by the Ca^{2+} channel antagonist mibefradil (Ro 40–5967). *Mol Pharmacol* **48:**540–549.

Chin LS, Havill, JH, and Rothwell RP (1979) Status epilepticus controlled by althesin infusion. *Anaesth Intensive Care* **7:**50–52.

Coulter DA, Huguenard JR, and Prince DA (1989a) Characterization of ethosuximide reduction of low-threshold calcium current in thalamic relay neurons. *Ann Neurol* **25:**582–593.

Coulter DA, Huguenard JR, and Prince DA (1989b) Specific petit mal anticonvulsants reduce calcium currents in thalamic neurons. *Neurosci Lett* **98:**74–78.

DeRiu PL, Mameli O, and Tolu E (1987) Comparison of the effects of althesin with diazepam and thiopental on cortical and subcortical epileptic activity in the rat. *Pharmacol Res Commun* **19:**59–68.

DeWaard M, Gurnett CA, and Campbell KP (1996) Structural, and functional diversity of voltage-activated calcium channels. In *Ion Channels* (Narahashi T, ed). pp 41–87, Plenum Press, New York,

ffrench-Mullen JM and Spence KT (1991) Neurosteroids block Ca^{2+} channel current in freshly isolated hippocampal CA1 neurons. *Eur J Pharmacol* **202:**269–272.

ffrench-Mullen JM, Danks P, and Spence KT (1994) Neurosteroids modulate calcium currents in hippocampal CA1 neurons via a pertussis toxin-sensitive G-protein coupled mechanism. *J Neurosci* **14:**1963–1977.

Gilron I and Coderre TJ (1996) Preemptive analgesic effects of steroid anesthesia with alphaxalone in the rat formalin test. *Anesthesiology* **84:**572–579.

Hamill OP, Marty A, Neher E, Sakmann B, and Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch* **381:**85–100.

Herrington J and Lingle CJ (1992) Kinetic and pharmacological properties of low voltage-activated Ca^{2+} current in rat clonal (GH_3) pituitary cells. *J Neurophysiol* **68:**213–232.

Herrington J, Stern RC, Evers AS, and Lingle CJ (1991) Halothane inhibits two components of calcium current in clonal (GH3) pituitary cells. *J Neurosci* **11:**2226–2040.

Hirano Y, Fozzard HA, and January CT (1989) Inactivation properties of T-type calcium current in canine cardiac Purkinje cells. *Biophys J* **56:**1007–1016.

Hodgkin AL, and Huxley AF (1952) A quantitative description of membrane current and its application to conduction and excitation in nerve. *J Physiol (Lond)* **117:**50–544.

Hu Y, Zorumski CF, and Covey DF (1993) Neurosteroid analogues: structure-activity studies of benz[*e*]indene modulators of $GABA_A$ receptor function. 1. The effect of 6-methyl substitution on the electrophysiological activity of 7-substituted benz[*e*]indene-3-carbonitriles. *J Med Chem* **36:**3956–3967.

Hu Y, Wittmer LL, Kalkbrenner M, Evers AS, Zorumski CF, and Covey DF (1997) Neurosteroid analogues. Part 5. Enantiomers of neuroactive steroids and benz[*e*]indenes: total synthesis, electrophysiological effects on $GABA_A$ receptor function and anesthetic actions in tadpoles. *J Chem Soc Perkin Trans* **1:**3665–3671.

Huguenard JR, and Prince DA (1994) Intrathalamic rhythmicity studied in vitro: nominal T-current modulation causes robust antioscillatory effects. *J Neurosci* **14:**5485–5502.

Huguenard JR and Prince DA (1992) A novel T-type current underlies prolonged Ca^{2+}-dependent burst firing in GABAergic neurons of rat thalamic reticular nucleus. *J Neurosci* **12:**3804–3817.

Huguenard JR (1996) Low-threshold calcium currents in central nervous system neurons. *Annu Rev Physiol* **58:**329–358.

Ikeda SR and Schofield GG (1989) Somatostatin blocks a calcium current in rat sympathetic ganglion neurons. *J Physiol (Lond)* **409:**221–240.

Lambert JJ, Belelli D, Hill-Venning C, and Peters JA (1995) Neurosteroids and $GABA_A$ receptor function. *Trends Pharm Sci* **16:**295–303.

Leresche N, Parri HR, Erdemli G, Guyon A, Turner JP, Williams SR, Asprodini E, and Crunelli V (1998) On the action of the anti-absence drug ethosuximide in the rat and cat thalamus. *J Neurosci* **18:**4842–4853.

Llinas R (1988) The intrinsic electrophysiological properties of mammalian neurons: insight into central nervous system function. *Science* **242:**1654–1664.

MacDonald RL and McLean MJ (1986) Anticonvulsant drugs: mechanisms of action. *Adv Neurol* **44:**713–736.

Matteson DR and Armstrong CM (1984) Properties of two types of calcium channels in clonal pituitary cells. *J Gen Physiol* **87:**161–182.

Mennerick S, Que J, Benz A, and Zorumski CF (1995) Passive and synaptic properties of hippocampal neurons grown in microcultures and mass cultures. *J Neurophysiol* **73:**320–332.

Mintz IM (1994) Block of Ca channels in rat central neurons by the spider toxin omega-Aga-IIIA. *J Neurosci* **14:**2844–2853.

Mishra SK and Hermsmeyer K (1994) Selective inhibition of T-type Ca^{2+} channels by Ro 40–5967. *Circ Res* **76:**144–148.

Nakashima Y, Todorovic SM, Covey DF, and Lingle CJ (1998) The anesthetic steroid (+)-3α-hydroxy-5α-androstane-17β-carbonitrile blocks N-, Q- and R-, but not L- and P-type, high voltage-activated Ca^{2+} current in hippocampal and dorsal root ganglion neurons of the rat. *Mol Pharmacol* **54:**559–568

Scroggs RS and Fox AP (1992) Calcium current variation between acutely isolated adult rat dorsal root ganglion neurons of different size. *J Physiol (Lond)* **445:**639–658.

Sear JW and Prys-Roberts C (1979) Plasma concentrations of alphaxalone during continuous infusion of althesin. *Br J Anesth* **51:** 861–865.

Sear JW (1996) Steroid anesthetics: old compound, new drugs. *J Clin Anesth* **8:**815–885.

Shapiro MS and Hille B (1993) Substance P and somatostatin inhibit calcium channels in rat sympathetic neurons via G protein pathways. *Neuron* **10:**11–20.

Solaro CR, Prakriya JP, Ding JP, and Lingle CJ (1995) Inactivating and non-inactivating Ca^{2+} and voltage-dependent K^+ current in rat adrenal chromaffin cells. *J Neurosci* **15:**6110–6123.

Spence KT, Plata-Salaman CR, and fFrench-Mullen JM (1991) The neurosteroids, pregnenolone and pregnenolone-sulfate but not progesterone, block Ca^{2+} currents in acutely isolated hippocampal CA1 neurons. *Life Sci* **49:**PL235-PL239.

Study RE (1994) Isoflurane inhibits multiple voltage-gated calcium current in hippocampal pyramidal neurons. *Anesthesiology* **81:**104–116.

Todorovic SM and Lingle CJ (1998) Pharmacological properties of T-type Ca^{2+} current in adult rat sensory neurons: effects of anticonvulsants and anesthetic agents. *J Neurophysiol* **79:**240–252.

Tsakiridou E, Bertollini L, De Curtis M, Avanzini G, and Pape H (1995) Selective increase in T-type calcium conductance in reticular thalamic neurons in a rat model of absence epilepsy. *J Neurosci* **15:**3110–3117.

Wittmer LL, Hu Y, Kalkbrenner M, Evers AS, Zorumski CF, and Covey DF (1996) Enantioselectivity of steroid-induced $GABA_A$ receptor modulation and anesthesia. *Mol Pharmacol* **50:**1581–1586.

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