0026-895X/98/030559-10$3.00/0

MOLECULAR PHARMACOLOGY, **54**:559–568 (1998).

The Anesthetic Steroid (+)-3α-Hydroxy-5α-androstane-17β-carbonitrile Blocks N-, Q-, and R-Type, but Not L- and P-Type, High Voltage-Activated Ca^{2+} Current in Hippocampal and Dorsal Root Ganglion Neurons of the Rat

YASUNORI M. NAKASHIMA,[1] SLOBODAN M. TODOROVIC, DOUGLAS F. COVEY, and CHRISTOPHER J. LINGLE

Departments of Anesthesiology (Y.M.N., S.M.T., C.J.L.) and Molecular Biology and Pharmacology (D.F.C.), Washington University School of Medicine, St. Louis, Missouri 63110

Received January 29, 1998; Accepted May 18, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

High voltage-activated (HVA) Ca^{2+} current (I_{Ca}) was recorded from neonatal rat hippocampal and adult rat dorsal root ganglion neurons. In both cell types, (+)-3α-hydroxy-5α-androstane-17β-carbonitrile [(+)-ACN], a neuroactive steroid, had no effect on nifedipine- (L-type) or ω-agatoxin IVA- (P-type) sensitive I_{Ca}. Selective blockade of N-type current with ω-conotoxin GVIA and of Q-type current with ω-conotoxin MVIIC indicated that (+)-ACN inhibits both N- and Q-type current components in both cell types. Current persisting after blockade of all other current components (R-type) was also sensitive to (+)-ACN. Half-blockade of (+)-ACN-sensitive HVA current occurred in the range of 3–25 μM, with N-type current somewhat more sensitive than Q- or R-type. The (+)-ACN enantiomer, (−)-ACN, and pregnanolone were somewhat less effective at inhibiting total HVA current than (+)-ACN, whereas several steroid analogs, including alfaxalone, were relatively ineffective at inhibiting total HVA current. Neither guanosine-5′-*O*-(2-thio)diphosphate nor guanosine-5′-*O*-(3-thio)triphosphate altered the ability of (+)-ACN to inhibit HVA current in dorsal root ganglion neurons, indicating that (+)-ACN acts directly on Ca^{2+} channels. The partial selectivity exhibited by (+)-ACN among different HVA current components suggests that manipulations of steroid analogues may be a useful strategy in the generation of more selective, more potent, small-molecular-weight HVA channel blockers.

Five distinguishable subtypes (L, N, P, Q, and R) of HVA I_{Ca} have been discerned in central nervous system neurons (Ishibashi *et al.*, 1995; Randall and Tsien, 1995; De Waard *et al.*, 1996). Many HVA currents share similarity in activation voltages and kinetic properties. As a consequence, identification of different current components has depended on particular peptide toxins, including ω-CgTx GVIA, ω-CmTx MVIIC, and ω-Aga IVA, that exhibit some degree of selectivity in their blocking actions. However, the usefulness of these peptides is somewhat limited for several reasons. First, some toxins block more than one type of HVA current, in some cases with overlapping potency (Randall and Tsien, 1995; McDonough *et al.*, 1996). For example, the inhibition by ω-CmTx MVIIC and ω-Aga-IVA of both P- and Q-type current has contributed to the suggestion that P- and Q-type channels may share similar molecular components (Stea *et al.*, 1994; De Waard *et al.*, 1996). Although careful consideration of the concentration dependence of block may allow differential effects on different current components to be discerned (McDonough *et al.*, 1996), overlap in sensitivities may limit the usefulness of such agents in evaluation of physiological roles. Second, the irreversible or slowly reversible nature of blockade (Randall and Tsien, 1995; McDonough *et al.*, 1996) of some currents by different peptides limits their usefulness in experiments in which the demonstration of reversibility of an effect would be desirable. Finally, although the potential physiological roles of different HVA current components can be evaluated in *in vitro* systems, peptides are not particularly suitable for *in vivo* evaluation of consequences of selective blockade of central nervous system HVA currents. Therefore, the availability of small-molecular-weight HVA current blockers with reasonable potency, selectivity, and reversibil-

This work was supported by National Institutes of Health Grant GM47969 (D.F.C. and C.J.L.).

[1] Current affiliation: Surgical Operating Theater, Kyushu University Hospital, Fukuoka, 812-8582, Japan.

ABBREVIATIONS: HVA, high voltage activated; (+)-ACN, (+)-3α-hydroxy-5α-androstane-17β-carbonitrile; I_{Ba}, Ba^{2+} current; I_{Ca}, Ca^{2+} current; I_{Cl}, Cl^- current; DMSO, dimethylsulfoxide; I-V, current-voltage; PS, pregnenolone sulfate; CgTx, conotoxin; Aga, agatoxin; DRG, dorsal root ganglion; GDPβS, guanosine-5′-*O*-(2-thio)diphosphate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ity would be of great value not only as tools for identification and definition of the physiological roles of particular HVA current components but also as a starting point for the development of a clinical pharmacology of central nervous system Ca^{2+} channels.

Steroids are interesting compounds for evaluation of pharmacological actions because they provide a rigid structural template on which both diverse and subtle structural variations can be introduced (Hu *et al.*, 1993; Han *et al.*, 1996). The anesthetic effects of some neurosteroids have been well documented, and these effects are thought to involve, to some extent, potentiation, gating, or both of $GABA_A$-mediated responses (Harrison and Simmonds, 1984; Wittmer *et al.*, 1996). However, qualitative differences in action among different steroids in both behavioral assays and clinical situations suggests that steroids may also exert effects on other ion channel targets (Kavaliers and Wiebe, 1987; Wieland *et al.*, 1995). In particular, some steroids have been reported to produce inhibitory effects on Ca^{2+} channels (ffrench-Mullen and Spence, 1991), although these effects have been reported to involve G protein mediation (ffrench-Mullen *et al.,* 1994). The availability of some new, particularly potent, anesthetic steroid analogs (Wittmer *et al.*, 1996) has prompted our interest in evaluating the ability of these steroids to inhibit different components of I_{Ca}. Here, using both cultured neonatal rat hippocampal neurons and acutely dissociated rat dorsal root ganglion neurons, we examined the sensitivity of different HVA current components to the novel neurosteroid (+)-ACN. The results show that although (+)-ACN does not affect L- and P-type current, it does block N-, Q-, and R-types of current with similar potency in both cell types. Thus, (+)-ACN exhibits partial selectivity in its blocking actions among I_{Ca} components. These effects of (+)-ACN seem to involve direct action on Ca^{2+} channels. Because the blocking effects of (+)-ACN on HVA I_{Ca} occur at concentrations outside the range of those effective at potentiating $GABA_A$ currents (Wittmer *et al.*, 1996), blockade of HVA I_{Ca} by (+)-ACN is unlikely to participate in its anesthetic effects. However, the partial selectivity in action of (+)-ACN in blocking Q- and N-type current over P- and L-type current suggests that manipulation of steroid structures is a promising strategy for the development of more potent, selective HVA Ca^{2+} channel antagonists.

Materials and Methods

Cell culture. Hippocampal neurons were cultured from 1–2-day-old albino rats as described previously (Rodgers-Neame *et al.*, 1992). Specifically, neurons were grown in microisland cultures, a procedure that minimizes arborizations associated with each neuron (Mennerick *et al.*, 1995). Furthermore, neurons were used after 3–7 days in culture (usually ≤5) to help minimize space-clamp problems associated with extensive processes.

DRG neurons were prepared from male rats (100–250 g; Sprague-Dawley) after anesthetization with halothane and decapitation. DRG (8–10) from thoracic and upper lumbar regions were dissected out and cells were dissociated as described previously (Todorovic and Lingle, 1998). Cells were kept at room temperature and used for electrophysiology within 4 hr from dissociation. For recordings from DRG neurons, neuronal cell bodies were plated onto a glass coverslip and placed in a 35-mm culture dish. All experiments were carried out at room temperature (~25°).

Solutions and drugs. The control Tyrode's solution contained 150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM HEPES, with pH adjusted to 7.4 with NaOH. The standard external solution used to isolate I_{Ba} contained 5 mM $BaCl_2$, 160 mM tetraethylammonium chloride, 10 mM HEPES, and 0.1 mM EGTA, with pH adjusted to 7.4 with tetraethylammonium hydroxide (standard osmolarity, 310 mOsM). The composition of standard internal solution was 110 mM Cs-methane sulfonate, 14 mM phosphocreatine, 10 mM HEPES, 9 mM EGTA, 5 mM Mg-ATP, and 0.3 mM Tris-GTP. The pH was adjusted to 7.3 with CsOH (standard osmolarity, 300 mOsM).

(+)-ACN, (−)-ACN (Hu *et al.*, 1993; Wittmer *et al.*, 1996), pregnanolone (3α-hydroxy-5β-pregnan-20-one; Sigma, St. Louis, MO), pregnenolone (3β-hydroxypregn-5-en-20-one; Sigma), pregnenolone sulfate (Sigma), (+)-3α-hydroxy-5α-pregnan-20-one, and alfaxalone (3α-hydroxy-5α-pregnane-11,20-dione; Sigma) were each dissolved in DMSO to make 10 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 <0.6% in these experiments; 1% DMSO did not affect I_{Ba} (data not shown, four experiments). At steroid concentrations of ≥30 μM, the steroids seem to crystallize out of solution, thus reducing the effective steroid concentration in solutions kept for >~10 min (data not shown, 10 experiments). To avoid this problem, all solutions containing concentrations of ≥30 μM were prepared just before each application (<30 sec). Solutions were applied to cells with the "Y-tube" technique, in which the previous solution in the tubing can be quickly flushed out and the fresh steroid solution quickly positioned for the next solution application.

ω -CgTx GVIA (RBI, Natick, MA; and Sigma), ω-CmTx MVIIC (RBI and Sigma), ω-Aga IVA (gift from Pfizer, Groton, CT), and $CdCl_2$ were dissolved in distilled water to make stock solutions of 0.5, 0.5, 0.2, and 200 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.

In some experiments, P- and Q-type I_{Ba} was blocked before initiation of recording (see Fig. 4). To accomplish this preblockade procedure, cells were incubated in an external solution containing 2 μM ω-CmTx MVIIC and 0.7 μM ω-Aga IVA for >30 min. After washing out this solution, recovery from block of P- and Q-type currents is quite slow (McDonough *et al.*, 1996), and block of P-type current is essentially irreversible over the time course of our experiments in the absence of strong depolarizing voltage steps (e.g., Mintz *et al.*, 1992). To ensure the persistence of blockade of P- and Q-type currents using this procedure, in some cells ω-CmTx MVIIC was reapplied just before the initiation of recording. In a few tests, ω-CmTx MVIIC was reapplied after recording was initiated. In such cases, additional block of I_{Ba} was minimal.

Methods for isolation of (+)-ACN-mediated inhibition of I_{Ba}. As described in the text, coincident activation of the $GABA_A$ receptor by (+)-ACN or other agents may confound efforts to define effects of (+)-ACN on I_{Ba}. The standard procedure used for all experiments on hippocampal neurons described here was to include 100 μM picrotoxinin (Sigma) and 50 μM bicuculline (Sigma) in all solutions. This solution had no direct effect on I_{Ba}, although in most cells, the combination of picrotoxinin/bicuculline blocked an outward current. Although the picrotoxinin/bicuculline combination fully blocked the ability of γ-aminobutyric acid to activate any current in the hippocampal neurons, in some cells, we observed a small direct activation of a presumed I_{Cl} by (+)-ACN in the presence of both antagonists (Fig. 1A). In the presence of 200 μM Cd^{2+}, the size of this potentially contaminating current was never larger than a few pA near 0 mV. A contaminating current of this magnitude does not influence any of the key observations or conclusions of this work. In DRG neurons, (+)-ACN did not activate any current in the presence of Cd^{2+} (five experiments).

Electrical recording technique. Single hippocampal or DRG neurons were voltage-clamped using the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). The resistance of the patch electrode was 2–4 MΩ when filled with the internal solution.

After a gigaohm seal was established, a strong negative pressure was briefly applied to the pipette interior to rupture the patch membrane. In hippocampal neurons, the experiments were initiated after >5 min, at which time the amplitude of I_{Ba} had stabilized. Typical series resistance (R_s) values at such times were ~10 MΩ with a typical whole-cell capacitance of 10 pF. In DRG neurons, for the set of smaller neurons (37 experiments), R_s = 7.8 ± 2.8 MΩ and C_m = 11.3 ± 3.1 pF. For larger neurons (36 experiments), R_s = 6.5 ± 2.2 MΩ and C_m = 26.8 ± 8.7 pF.

To record I_{Ba}, square pulses to −10 mV for 25 msec were applied every 15 or 20 sec. In hippocampal neurons, a holding potential (V_h) of −80 mV was used; for DRG neurons, the holding potential was −60 mV to abolish activation of T-type current (e.g., Todorovic and Lingle, 1998). For I-V relationships, a ramp pulse (dV/dt = ± 1 V/sec) was applied from V_h of −80 mV, and voltage extremes were set to −80 and +100 mV. The I-V relations were measured from the depolarizing portion of the ramp pulses. The current and voltage were recorded using a patch-clamp amplifier (Axopatch-1C, Axon Instruments, Foster City, CA) and pClamp software (Axon Instruments) and stored in the computer.

Because these cultured hippocampal neurons have long processes, voltage-clamp conditions were less than ideal, even if neurons with shorter processes are selected. Because of this, rapid components of current, such as tail currents, are not likely to reflect the true amplitude and time course of I_{Ba} behavior. However, all measurements of amplitudes from holding, peak, and steady state currents are made at time points sufficient to ensure reasonably well-clamped current conditions. Example traces from hippocampal neurons typically show currents after subtraction of traces obtained in Cd^{2+}. For currents from DRG neurons, traces show raw currents without leak subtraction. No correction was made for R_s.

Statistics. The concentration-response curves for the percent block of I_{Ca} by (+)-ACN were drawn according to the equation

$$I(C) = I_{max} \cdot C^{nH}/(C^{nH} + IC_{50}{}^{nH}) \quad (1)$$

where I(C) and I_{max} are the observed and maximum blocking percentages of I_{Ca}, and C is the (+)-ACN concentration. IC_{50} and n_H denote the concentration producing 50% block and the Hill coefficient, respectively.

All data are shown as mean ± standard deviation.

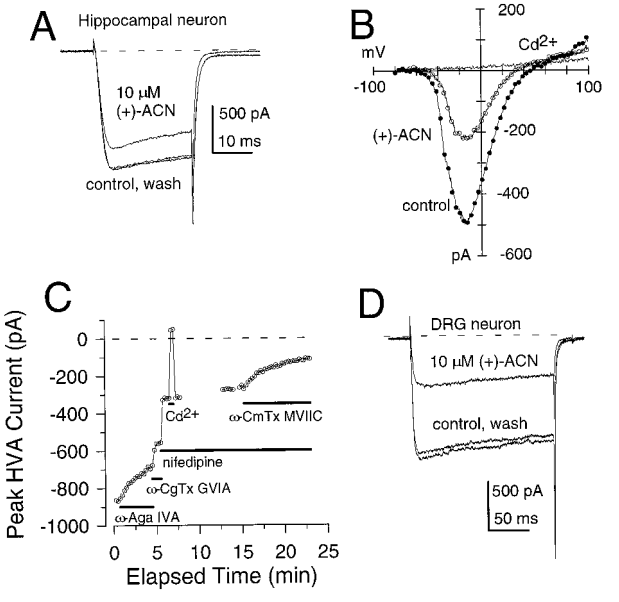

Fig. 1. (+)-ACN reduces HVA I_{Ca} in hippocampal and DRG neurons. A, *Traces* show currents elicited by the application of a 25-msec square pulse to −10 mV from a holding potential (V_h) of −80 mV. Leak current was corrected by subtracting currents elicited in 0.2 mM Cd^{2+}. External solution contained 50 μM bicuculline (*BIC*) and 100 μM picrotoxinin (*PTX*). B, Curves show I-V relations obtained by the application of a ramp pulse as described in Materials and Methods with V_h of −80 mV. *Curves*, I-V relationships for control saline, 30 μM (+)-ACN, and 0.2 mM Cd^{2+}. All solutions contained 50 μM bicuculline and 100 μM picrotoxinin. C, Peak HVA current amplitude is plotted as a function of elapsed time for a recording from a hippocampal neuron. HVA current was elicited as in A with a depolarizing step applied every 15 sec. ω-Aga IVA (0.1 μM), ω-CgTx (1 μM), nifedipine (5 μM), ω-CmTx MVIIC (1 μM), and Cd^{2+} (0.2 mM) were applied during the periods indicated (*bars*). The external solution contained 5 mM Ba^{2+} as the charge carrier. D, *Traces* show total I_{Ba} elicited by a 200-msec step to −10 mV with V_h of −60 mV in an acutely isolated DRG neuron. 10 μM (+)-ACN markedly inhibits HVA current.

Results

(+)-ACN reduces HVA I_{Ba} in cultured hippocampal and acutely dissociated DRG neurons. A number of compounds reported to have effects on I_{Ca} also are known to produce direct gating of $GABA_A$ receptors; this includes a number of anesthetics such as propofol, isoflurane, and pentobarbital (Gross and MacDonald, 1988; Jones *et al.*, 1992; Hara *et al.*, 1994; Olcese *et al.*, 1994; Study, 1994). The steroid, (+)-ACN, also activates $GABA_A$-gated Cl^- channels in rat hippocampal neurons at concentrations in excess of 1 μM (Wittmer *et al.*, 1996). We therefore were concerned that despite the usual solutions used to isolate I_{Ba}, coincident activation of I_{Cl} might contaminate records of I_{Ba}.

To address this problem, we used a combination of 100 μM picrotoxinin with 50 μM bicuculline. Although each compound alone was found to be insufficient at fully reducing the activation of $GABA_A$ current by steroids, the combination of these two agents essentially removed most I_{Cl} activation by (+)-ACN (data not shown, see Materials and Methods). Fig. 1A shows original I_{Ba} traces from a hippocampal neurons activated by a 25-msec command step to −10 mV from −80 mV, before, during, and after the application of 10 μM (+)-ACN. At concentrations of ≥10 μM, (+)-ACN consistently reduces the magnitude of inward current. To illustrate that these traces are not confounded by the simultaneous activation of an outward I_{Cl}, Fig. 1B shows I-V curves obtained from voltage ramps applied before and after the application of (+)-ACN in the presence of the bicuculline/picrotoxinin cocktail. In addition, a third trace was generated in the presence of 200 μM Cd^{2+}. The similar intersection of current traces obtained before and during 30 μM (+)-ACN with that obtained during blockade of I_{Ba} by Cd^{2+} indicates that the I-V relationship is not significantly contaminated by the coincident activation of I_{Cl}. The activation of I_{Cl} by (+)-ACN would have substantially shifted the apparent I_{Ba} reversal potential to much more negative potentials. Thus, Fig. 1, A and B, shows that (+)-ACN produces appreciable inhibition of HVA I_{Ba} in hippocampal neurons.

I_{Ba} in these hippocampal cells consists of multiple, pharmacologically distinct components (Ishibashi *et al.*, 1995). Fig. 1C demonstrates the pharmacological identification of specific subtypes of I_{Ba} in these cells. We observed that 1 μM ω-CgTx GVIA, 0.1 μM ω-Aga IVA, and 5 μM nifedipine, relatively selective blockers of N-, P-, and L-type Ca^{2+} channels, blocked 29 ± 8%, 11 ± 7%, and 26 ± 5% of the total I_{Ba}, respectively (13 experiments). These fractions are comparable to earlier work on hippocampal CA1 neurons, in which N, P-, L-, Q-, and R-type current components comprised 27 ± 3%, 13 ± 1%, 38 ± 4%, 9 ± 2%, and 13 ± 2% of total I_{Ba},

respectively (Ishibashi *et al.*, 1995). In addition, in our experiments, after blockade of N-, P-, and L-type -, ω-CmTx MVIIC (1–2 μM) blocked an additional 18 ± 4% of I_{Ca} (four experiments), whereas ~16% of Cd^{2+}-sensitive current was resistant to all Ca^{2+} channel blockers. We will refer to this resistant current as R-type (Randall and Tsien, 1995), although the identity of such currents remains unclear and may to some extent involve residual unblocked channels of the other Ca^{2+} channel subtypes.

To provide an additional test of the effects of (+)-ACN on HVA I_{Ca}, the effects of (+)-ACN on HVA current in acutely dissociated DRG neurons also were examined. As shown in Fig. 1D, (+)-ACN markedly reduces total I_{Ba} in these cells. The identity of different HVA current components in DRG neurons depends to some extent on the diameter of the underlying cells (Scroggs and Fox, 1992). Small-diameter neurons typically seem to be selectively enriched in L- and N-type HVA currents (Scroggs and Fox, 1992). In contrast, larger DRG neurons express a larger portion of non-L, non-N current, which has been reported to be of P-type (Mintz *et al.*, 1992), Q-type (Rusin and Moises, 1995), and a component unblocked by any known blocker (Rusin and Moises, 1995), presumably R-type. Therefore, for examination of the effects of (+)-ACN on L- and N-type current DRG neurons, we selected smaller-diameter cells (generally <21 μm), whereas larger cells (>23 μm) were used for examination of the effects of (+)-ACN on other currents.

L- and P-type I_{Ba} are insensitive to (+)-ACN. To determine whether (+)-ACN may selectively block particular components of I_{Ba}, the amplitude of current blocked by 30 μM (+)-ACN was compared before and after blockade of particular HVA current components. Fig. 2A plots the absolute amplitude of current activated by depolarizing steps to −10 mV during the time course of an experiment. Fig. 2B shows the original I_{Ba} traces. (+)-ACN (30 μM) blocked ~150 pA of I_{Ba}. After blockade of ~60 pA of current by the addition of 0.1 μM ω-Aga IVA to the external solution, 30 μM (+)-ACN still blocked ~150 pA of current. In 15 hippocampal cells, (+)-ACN did not block P-type current. This suggests that under conditions that result in complete blockade of P-type current, the current blocked by (+)-ACN is unaffected. Similar results were obtained in DRG neurons in which L- and N-type currents were blocked by nifedipine and ω-CgTx GVIA, respectively. As shown in Fig. 2C, 30 μM (+)-ACN blocks ~1000 pA of current in this DRG neuron. After blockade of ~300 pA of I_{Ba} by 0.1 μM ω-Aga IVA, 30 μM (+)-ACN still blocks ~1000 pA of current. Fig. 2D displays traces indicating that (+)-ACN blocks the same amount of current before and after blockade of current by ω-Aga IVA. The lack of effect of ω-Aga IVA on blockade by (+)-ACN was observed in seven DRG neurons (also see Fig. 5C).

Similarly, 5 μM nifedipine does not reduce the amplitude of the current blocked by (+)-ACN (Fig. 3A). This indicates that the current sensitive to (+)-ACN is not L-type I_{Ba}. The lack of effect of nifedipine on the amplitude of current blocked by (+)-ACN was observed in 13 cells. Thus, the (+)-ACN block of HVA I_{Ba} does not seem to involve an effect on either L- or P-type currents. Similarly, in Fig. 3B, 5 μM nifedipine does not change the amplitude of current blocked by (+)-ACN in a DRG neuron. The lack of effect of nifedipine on blockade by (+)-ACN was observed in eight DRG neurons.

ω -CgTx GVIA-blockable current (N-type) is blocked by (+)-ACN. To examine the effects of (+)-ACN on N-type current in hippocampal neurons, P- and Q-type currents were first minimized by prior blockade with ω-Aga IVA and ω-CmTx MVIIC as described in Materials and Methods. ω-CmTx MVIIC also blocks N-type current, but the block of N-type is rapidly reversible. Nifedipine then was applied continuously to remove L-type current. Fig. 4A illustrates the effect of (+)-ACN after blockade of P-, Q-, and L-type current. In this cell, 10, 30, and 60 μM (+)-ACN were sequentially applied, and 60 μM (+)-ACN blocked ~200 pA of current, which was almost all of the remaining Cd^{2+}-blockable current. After blockade of ~150 pA of N-type current with ω-CgTx GVIA, 60 μM (+)-ACN blocked <50 pA of current. Representative traces from this experiment are shown (*right*), indicating that in this cell almost all the ω-CgTx GVIA-blockable current also is blocked by (+)-ACN.

Can any inferences about the concentration dependence of N-type current block by (+)-ACN be made from this sort of experiment? In this cell, the amount of I_{Ba} blocked by ω-CgTx GVIA was large relative to that persisting after block of N-type current. Thus, block of the ω-CgTx GVIA-resistant current by 60 μM (+)-ACN is only a small portion of the block produced by 10 and 30 μM (+)-ACN before ω-CgTx GVIA

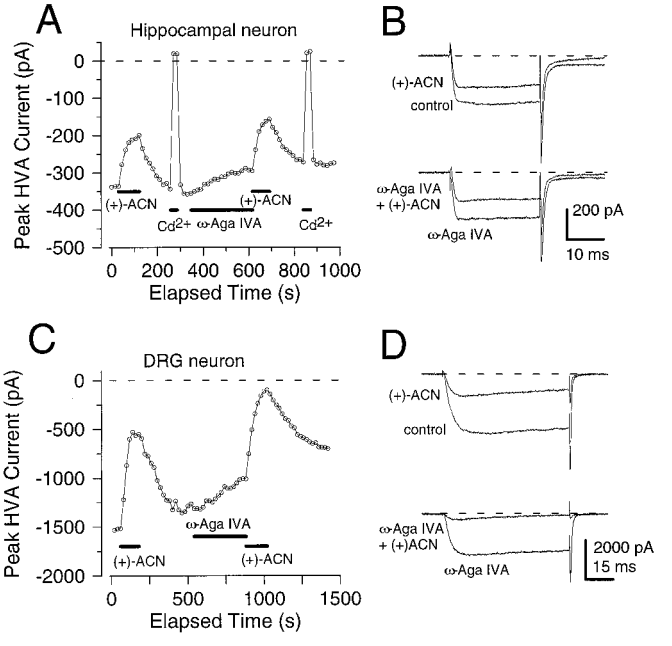

Fig. 2. P-type current is not blocked by (+)-ACN. A, Peak HVA current amplitude is plotted as a function of experimental time as in Fig. 1C for a neonatal rat hippocampal neuron. Depolarizations were applied every 15 sec. (+)-ACN (30 μM), Cd^{2+} (0.2 mM), and ω-Aga IVA (0.1 μM) were applied during the periods indicated (*bars*). Block of HVA current by ω-Aga IVA does not alter blockade by (+)-ACN. B, *Traces* used to generate the plot shown in A are displayed. *Top*, blockade by (+)-ACN of total HVA current. *Bottom*, the blocking effect of (+)-ACN after blockade of current by 0.1 μM ω-Aga IVA. Note that the amount of current blocked by (+)-ACN did not change before and after P-type current blockade. C, Peak HVA current amplitude in a large DRG neuron (C_m = 28 pF; R_s = 4 MΩ) is shown as a function of elapsed time. In this cell, currents were elicited in the continuous presence of 5 μM nifedipine and after blockade of N-type current by 1 μM ω-CgTx GVIA. (+)-ACN (30 μM) and 0.1 μM ω-Aga IVA were applied during the periods indicated (*bars*). D, *Traces* used to generate the plot shown in C are displayed. (+)-ACN blocks the same amount of HVA current both before (*top*) and after (*bottom*) blockade by 0.1 μM ω-Aga IVA.

application. Thus, in this cell, the amount of current blocked by 10 μM (+)-ACN is somewhat more than half of the total ω-CgTx GVIA-blockable current, with some uncertainty due to some residual block by 60 μM (+)-ACN or perhaps current run-down. Thus, 10 μM (+)-ACN may be blocking a little more than half of the N-type current in this cell.

A second example of block of N-type current in a hippocampal neuron is shown in Fig. 4B. Again, P-, Q-, and L-type currents were blocked as above; 10, 30, and 60 μM (+)-ACN then were applied sequentially, and the amplitude of the current blocked by 60 μM (+)-ACN was reduced from ~750 pA to ~250 pA after blockade of N-type current by ω-CgTx GVIA. In this cell, presumed R-type current is a larger fraction of the total I_{Ba}. Taking into account that a larger portion of the current blocked by 10, 30, and 60 μM (+)-ACN is non-N-type current, in this cell, it seems that 10 μM (+)-ACN is blocking less than half of the total ω-CgTx GVIA-sensitive current. Overall, in seven cells studied with this method, the absolute amount of combined N/R-type current blocked by (+)-ACN was markedly reduced after the application of ω-CgTx GVIA. A substantial portion of the current blocked by (+)-ACN was removed by the irreversible blocking action of ω-CgTx GVIA on N-type current. Because both the N and the non-N component are sensitive to (+)-ACN, it is difficult to assess the relative concentration dependence of the N-type current in hippocampal neurons. However, it is clear that 10 μM (+)-ACN can block a substantial fraction of N-type current. Furthermore, the persistent R-type current also exhibits its sensitivity to (+)-ACN.

In DRG neurons, small-diameter neurons express predominantly L- and N-type HVA current (Scroggs and Fox, 1992). Selection of such cells allows relatively unambiguous examination of N-type current after blockade of L-type current. In the DRG neuron shown in Fig. 4C, 30 μM (+)-ACN blocks almost 2800 pA of HVA current. After blockade of L-type current with nifedipine, the amplitude of the current blocked by (+)-ACN is unchanged. Subsequent blockade of N-type current with ω-CgTx GVIA almost completely blocks any inhibitory effect of (+)-ACN on the residual I_{Ba}. Thus, as shown in Fig. 4C (*right traces*), in a cell with predominantly N-type current, removal of N-type current by ω-CgTx GVIA

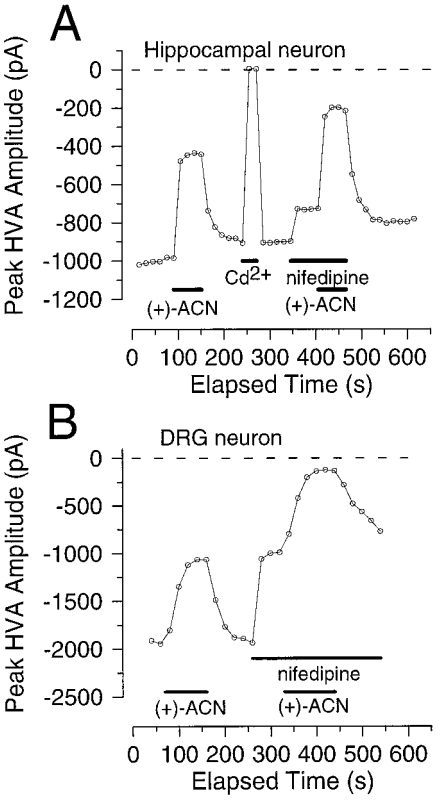

Fig. 3. L-type current is not blocked by (+)-ACN. A, Peak HVA current amplitude is plotted versus elapsed time for a hippocampal neuron. (+)-ACN (30 μM), Cd^{2+} (0.2 mM), and nifedipine (5 μM) were applied during the periods indicated (*bars*). The amount of current blocked by (+)-ACN did not change before and after block of L-type current by nifedipine. B, Peak HVA current versus elapsed time is plotted for a DRG neuron. Nifedipine (5 μM) did not alter the amount of current blocked by 30 μM (+)-ACN.

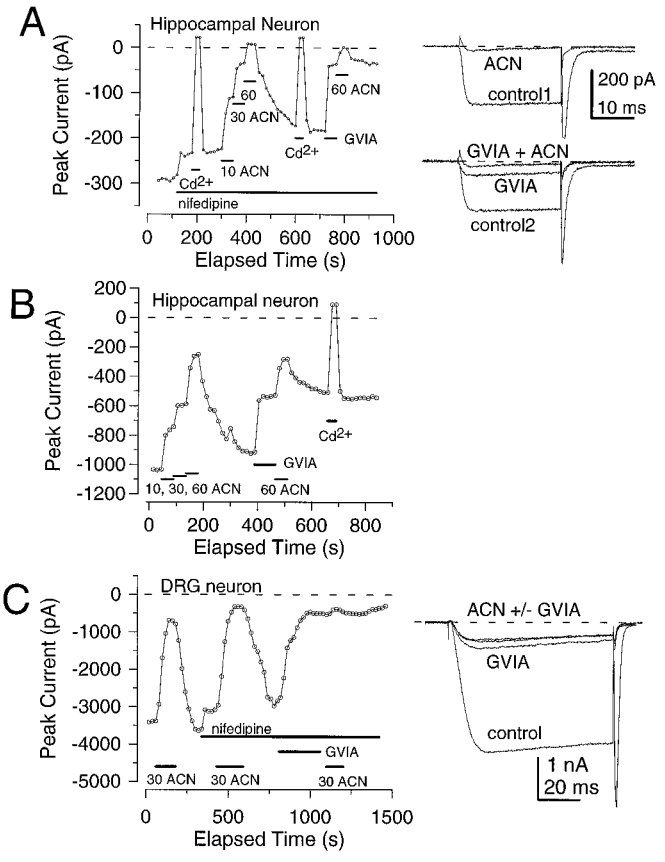

Fig. 4. N-type currents are blocked by (+)-ACN. A, Peak HVA current amplitude is plotted over the course of an experiment. L-, P-, and Q- type currents were already blocked by coapplication of nifedipine (5 μM) and preapplication of ω-Aga IVA (0.7 μM) and ω-CmTx MVIIC (2 μM). Various concentrations of (+)-ACN (10, 30, and 60 μM) were applied during the periods indicated (*bars*). ω-CgTx GVIA (1 μM) and Cd^{2+} (0.2 mM) were also applied as indicated (*bars*). (+)-ACN blocked the remaining current (N- and R-type) in a concentration-dependent fashion, and blockade by (+)-ACN was substantially reduced after blockade of N-type current by ω-CgTx GVIA. *Right*, *traces* show the block of HVA current by (+)-ACN before (*top*) and then after (*bottom*) block of N-type current by ω-CgTx GVIA. B, Time course of block of peak HVA current is plotted for a cell in which the remaining R-type current is substantial relative to the total N-type current. Applications of (+)-ACN, ω-CgTx GVIA, and Cd^{2+} are indicated (*bars*), with concentrations as in A. As in A, a large portion of (+)-ACN-blockable current is N-type current. C, Time course of block of peak HVA current is plotted for a DRG neuron. (+)-ACN (30 μM) was applied as indicated. After blockade of L-type current by nifedipine (5 μM), (+)-ACN blocks the same amount of current. ω-CgTx GVIA almost totally blocks the (+)-ACN-blockable current. *Right*, *traces* show the block by (+)-ACN before and after blockade of N-type current.

almost completely removes the (+)-ACN-blockable current. N-type current in DRG neurons seems to be strongly blocked by (+)-ACN.

An ω-CmTx MVIIC-sensitive current is also blocked by (+)-ACN. A similar strategy was used to assess whether (+)-ACN might produce inhibition of Q-type current. After preblockade in a hippocampal neuron of L-, N-, and P-type currents with nifedipine, ω-CgTx GVIA, and ω-Aga-IVA, respectively, sequential applications of 10, 30, and 60 μM (+)-ACN showed that up to ~70% of the remaining Q/R-type current could be blocked (Fig. 5A). Fig. 5B (*top traces*) shows representative currents before (+)-ACN application and during the application of 60 μM (+)-ACN. Subsequent application of 2 μM ω-CmTx MVIIC then blocked almost 200 pA of current, resulting in a marked reduction in the amount of current that was subsequently blocked by (+)-ACN (Fig. 5B). In nine of nine cells, the absolute amount of combined Q/R-type current inhibited by (+)-ACN was markedly reduced after the application of ω-CmTx MVIIC. This result shows

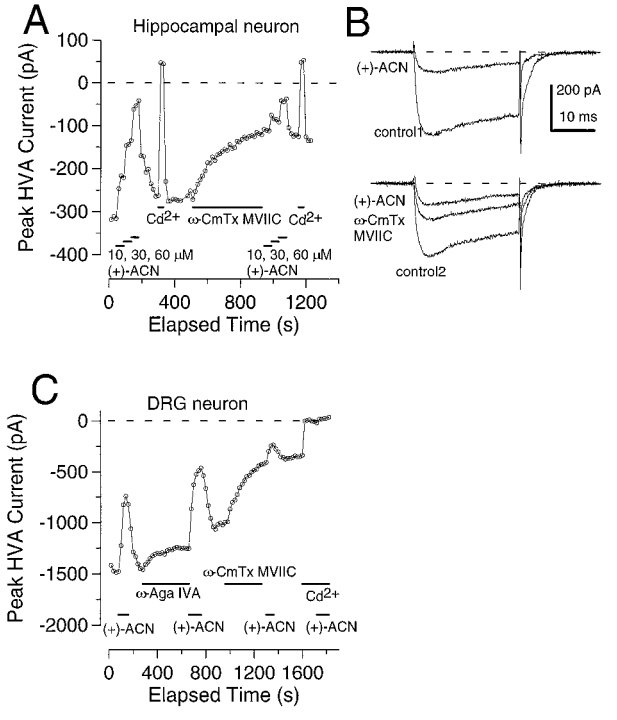

Fig. 5. Q- and R-type currents are blocked by (+)-ACN. A, Time course of peak HVA current is plotted for a hippocampal neuron. L-, N-, and P-type currents were already blocked by coapplication of nifedipine (5 μM) and preapplication of ω-CgTx (1 μM) and ω-Aga IVA (0.7 μM). Three sequential concentrations of (+)-ACN (10, 30, and 60 μM) were applied during the periods indicated (*bars*). ω-CgTx MVIIC (2 μM) and Cd^{2+} (0.2 mM) were also applied as indicated. (+)-ACN blocked the remaining Q- and R-type current in a concentration-dependent fashion. Percent block of HVA current by (+)-ACN was less after blockade of Q-type current by ω-CgTx MVIIC. (+)-ACN also blocked the remaining current (R-type) in a concentration-dependent manner. B, *Traces* show the effect of 60 μM (+)-ACN on HVA current before (*top*) and after (*bottom*) after blockade of Q-type current by ω-CmTx MVIIC. Traces obtained in Cd^{2+} were subtracted in each case. C, Time course of block of HVA current is displayed for a DRG neuron. This cell was continuously bathed in 5 μM nifedipine, and 1 μM ω-CgTx GVIA was briefly applied to block N-type current. 30 μM (+)-ACN was applied as indicated (*bars*). ω-Aga IVA (0.1 μM) did not alter the amount of current blocked by (+)-ACN (similar to Fig. 2). Application of 2 μM ω-CmTx MVIIC blocked a sizable portion of the current and markedly reduced the size of the current blocked by (+)-ACN. (+)-ACN has no effect in DRG neurons after complete blockade of I_{Ca} by 0.2 mM Cd^{2+}.

that most or all of the current blocked by ω-CmTx MVIIC is also blocked by (+)-ACN, after prior blockade of L-, N-, and P-type currents. We do not know how much of the current persisting after ω-CmTx MVIIC application is residual unblocked Q- or R-type current.

A similar experiment is shown in Fig. 5C for a DRG neuron in which N-type current was previously blocked by ω-CgTx GVIA, and 5 μM nifedipine was continuously applied. (+)-ACN blocks ~700 pA of the remaining current in this cell. After blockade of ~250 pA of current by ω-Aga IVA, (+)-ACN still blocks a similar amount of I_{Ba}. However, after a ~5-min application of 2 μM ω-CmTx MVIIC, which blocked ~600 pA of current, (+)-ACN only blocked ~150–200 pA of additional current. This suggests that (+)-ACN blocks a Q-type current (i.e., current sensitive to ω-CmTx MVIIC that persists after application of 100 nM ω-Aga IVA).

Finally, the persistence of some (+)-ACN-sensitive current after block of all other current components in both DRG and hippocampal neurons again argues that R-type current also is sensitive to (+)-ACN. However, it is possible that in experiments in which P- and Q-type currents were preblocked by ω-Aga IVA and ω-CmTx MVIIC, the residual, (+)-ACN-sensitive current might reflect some recovery from the toxin block of P- and Q-type current. To exclude this possibility, in several hippocampal cells an additional application of ω-CmTx MVIIC was used to show that little recovery from blockade of P- and Q-type current had occurred. Furthermore, the remaining (+)-ACN-sensitive current was larger in amplitude than any residual ω-CmTx MVIIC-sensitive component of current, indicating that R-type current is, in fact, blocked by (+)-ACN. Similarly, in DRG neurons, (+)-ACN blocked residual current in all 10 neurons where N-, L-, P-, and Q-type currents were pharmacologically removed.

Concentration-dependence of block of I_{Ba} by (+)-ACN. Because we know of no selective inhibitors of R-type current, for the hippocampal neurons it is not currently possible to define the pharmacological sensitivity of isolated Q- or N-type current to (+)-ACN in the absence of concomitant R-type current activation. As a consequence, three separate concentration-response curves were generated to assess the concentration dependence of (+)-ACN inhibition. First, the concentration-dependent blockade of I_{Ba} by (+)-ACN was evaluated after simultaneous blockade of L-, P-, N-, and Q-type currents. This allows definition of the sensitivity of R-type current to (+)-ACN (Fig. 6A). Second, the effects of (+)-ACN on combined Q/R-type current were determined (Fig. 6B). Third, the effects of (+)-ACN on combined N/R-type current were defined (Fig. 6C). It should be kept in mind that each of these concentration-response curves is limited by assumptions about the pharmacological specificity of various blockers and, when the preblocking procedure was used, by the extent to which block persists. However, these curves provide an important first step in clarifying the relative specificity of (+)-ACN in blocking native I_{Ca}.

The concentration-response curves for blockade of presumed R-type current were largely indistinguishable between hippocampal and DRG neurons (Fig. 6A). Blockade of R-type current in hippocampal neurons occurred with an IC_{50} value of 28.6 ± 4.5 μM, whereas blockade of R-type current in DRG neurons occurred with an IC_{50} value of 21.0 ± 2.4 μM. Blockade of Q- and R-type current occurred with an IC_{50} value of 23.4 ± 1.6 μM in hippocampal neurons

and an IC_{50} value of 16.0 ± 2.8 μM in DRG neurons (Fig. 6B). Blockade of N- and R-type current occurred with an IC_{50} value of 19.5 ± 1.7 μM in hippocampal neurons, whereas block of the better isolated, largely N-type current in DRG neurons occurred with an IC_{50} value of 3.6 ± 0.6. In each case, maximal block seems to be near 100%, although the true level of maximal block is uncertain. The combined concentration-response curves suggest that R-type current may be somewhat less sensitive to blockade by (+)-ACN than the other two current components; this also is suggested by the records in Figs. 5 and 6. Specifically, the fractional block by 60 μM (+)-ACN is smaller after blockade of either N-type (Fig. 5) or Q-type (Fig. 6) current. The apparent discrepancy between block of N-type current in DRG cells and N/R-type current in hippocampal cells may be smaller than is suggested by Fig. 6C. As pointed out earlier regarding the records in Fig. 4A, in a hippocampal cell in which N-type current is the major contributor to the combined N/R-type current, 10 μM (+)-ACN seems to block at least half of the ω-CgTx GVIA-sensitive current. This suggests the N-type current sensitivity in hippocampal cells is not too dissimilar from that in DRG neurons. One explanation for the differences in Fig. 6C is that the lower affinity IC_{50} value of the combined N/R concentration-response curve for hippocampal neurons may be skewed by a large contribution of R-type current in most cells and the weaker sensitivity of the R-type current (Fig. 6A).

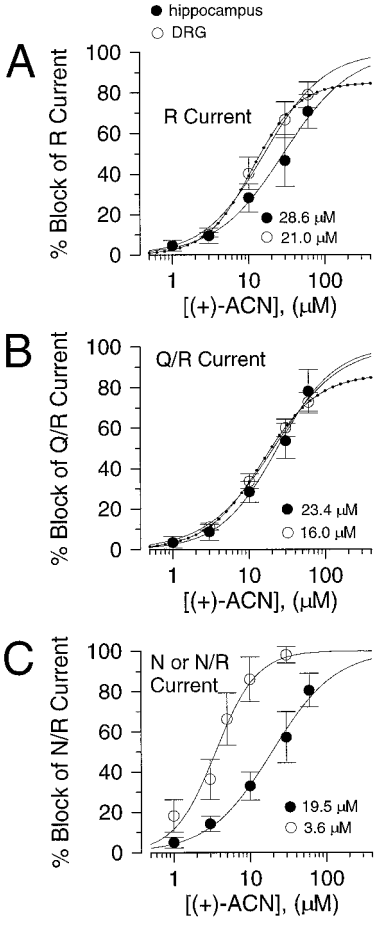

Fig. 6. Concentration-dependent blockade of R-, Q/R-, and N/R-type currents by (+)-ACN in hippocampal and DRG neurons. A–C, For hippocampal neurons, the amount of block was determined from the reduction of peak current relative to the current blocked by Cd^{2+}. For DRG neurons, block was determined relative to the 0 current level. *Solid and dotted curves*, drawn according to eq. 1. *Points,* average of 5–10 neurons; *error bars*, mean ± standard deviation. A, R-type currents were isolated after block of L-, N-, P-, and Q-type currents. For hippocampal R-type current, with maximal block constrained to 100%, the IC_{50} value was 28.6 ± 2.6 (n = 1.0, where n is the Hill coefficient, as in eq. 1) for DRG neurons, with maximal block constrained to 100%, the IC_{50} value was 21.0 ± 2.4 μM (n = 1.0); with no constraint on maximal block (86.5 ± 6.7%), the IC_{50} value was 14.8 ± 2.7 (n = 1.2). B, The concentration dependence of block of Q/R-type current by (+)-ACN was determined after block of N-, L-, and P-type currents (as in Fig. 5). With maximal block constrained to 100%, for Q/R-type current, the IC_{50} value was 23.4 ± 1.6 μM (n = 1.2) in hippocampal neurons and 16.0 ± 2.8 μM (n = 1.1) in DRG neurons. For DRG neurons with no constraint on maximal block (85.1 ± 19.1%), IC_{50} = 11.2 ± 6.0 (n = 1.4). C, Blockade of N- and N/R-type current was assessed as shown in Fig. 4. For hippocampal neurons, the IC_{50} value was 19.5 ± 1.7 μM (n = 1.0). For block of the largely N-type current in DRG neurons, the IC_{50} value was 3.6 ± 0.6 μM (n = 1.6).

Inhibition of HVA current by (+)-ACN is not G protein mediated. Several aspects of the block of different HVA current components by (+)-ACN seem inconsistent with the involvement of a G protein-mediated pathway. First, (+)-ACN seems able to block N-, Q-, and R-type currents almost completely. Second, the amount of inhibition seems to be quite stable over repeated applications of (+)-ACN. Third, we have observed none of the temporal alterations of HVA current time course often observed for some types of G protein-mediated inhibition (Mintz and Bean, 1993; Ikeda, 1996). To address this issue more directly, we examined the ability of alterations of G protein-mediated signaling to interfere with the inhibitory action of (+)-ACN on HVA current in DRG cells.

In one set of experiments, inhibition of G protein-mediated signaling was accomplished by introduction of GDPβS into the recording pipette. After activation of G proteins, GDPβS, an antagonist of G protein activation (Eckstein *et al.*, 1979; Holz *et al.*, 1986), will displace other guanine nucleotides from the GDP/GTP binding site on the G protein α subunit. Subsequent activation of G protein coupled receptors will fail to result in G protein activation. Such an experiment is illustrated in Fig. 7, in which inclusion of GDPβS results in abolition of a muscarinic receptor-mediated inhibition of HVA current (Fig. 7B) without affecting inhibition produced by 10 μM (+)-ACN (Fig. 7, A and B). In these experiments, GDPβS also produced a characteristic run-up of HVA current, perhaps indicative of the removal of some tonic inhibition of HVA current under our experimental conditions. In these experiments, because small DRG neurons were used and L-type currents were blocked with 5 μM nifedipine, the HVA current was predominantly of N-type. In the presence of 2 mM GDPβS, 10 μM (+)-ACN inhibited 80.5 ± 6.1% (mean ± standard deviation; four experiments) of the HVA current. With 300 μM GTP in the pipette, 10 μM (+)-ACN inhibited 83 ± 10% (mean ± standard deviation; five experiments) of the HVA current persisting after blockade of L-type current.

In a second set of experiments, the recording pipette contained 100 μM GTPγS. With GTPγS, HVA current exhibited a rapid run-down to ~10–20% of its initial level as seen in other systems (Ikeda and Schofield, 1989). Once this steady state level of HVA current was achieved, 10 μM (+)-ACN still blocked 76.3 ± 6.6% (mean ± standard deviation; six experiments) of the residual HVA current. In two of these six cells, ω-CgTx GVIA was applied after the reduction in HVA current by GTPγS had approached a steady state level (Fig. 7C). In both cases, most of the residual current blocked by (+)-

ACN was also blocked by ω-CgTx GVIA, indicating that N-type current is still blocked by (+)-ACN after G protein activation.

Initial structure-activity relationships for blockade of I_{Ca} by steroids. There have been other reports of blocking effects of steroids on I_{Ca} (ffrench-Mullen and Spence, 1991; Spence *et al.*, 1991; ffrench-Mullen *et al.*, 1994). To assess to what extent the effects of (+)-ACN may resemble the actions of these other steroids, we examined several steroid analogs, including some previously reported to block I_{Ba}. To provide a simple comparison of the relative effectiveness of these agents, the ability of 30 μM concentration of each compound to inhibit total I_{Ba} was determined and compared in the same cells with the ability of 30 μM (+)-ACN to inhibit total I_{Ba}. These results are summarized in Table 1. (−)-ACN, the enantiomer of (+)-ACN, was somewhat weaker than (+)-ACN in inhibiting total HVA I_{Ba}. The anesthetic steroid pregnanolone also exhibited an effectiveness comparable to (+)-ACN in inhibiting total I_{Ba}. In contrast, the anesthetic steroids alfaxalone and (+)-3α-hydroxy-5α-pregnan-20-one produced only small effects on total I_{Ba} at 30 μM. Furthermore, we observed that 30 μM pregnenolone was essentially ineffective at producing inhibition of HVA I_{Ba}. In contrast, 30 μM PS was observed to produce a small, but consistent, increase in total HVA I_{Ba}. Voltage-ramp elicited currents showed that the increase produced by PS was not associated with any change in outward leak current or change in the apparent reversal potential for I_{Ba} (data not shown). Thus, the results suggest that PS may produce a direct enhancement of I_{Ba}.

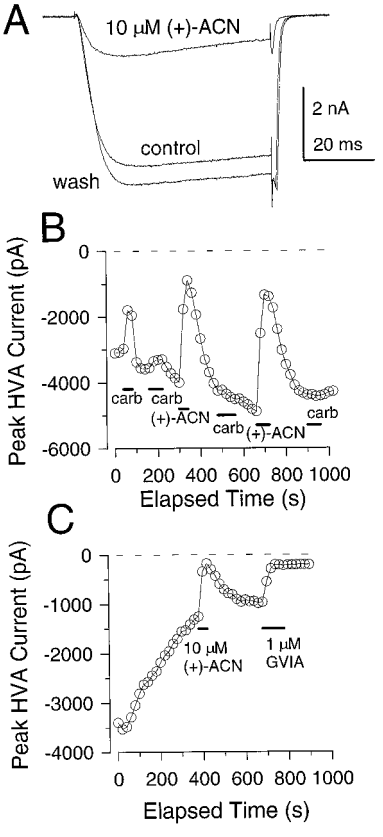

Fig. 7. Inhibition of G protein-mediated pathways does not alter inhibition by (+)-ACN of HVA current in DRG neurons. A, HVA current was recorded from a smaller DRG neuron during continuous block of L-type current with nifedipine. The pipette solution contained 2 mM GDPβS and no GTP. *Traces*, currents activated by steps to −10 mV from a holding potential of −60 mV before, during, and after application of 10 μM (+)-ACN at a time when 10 μM carbachol no longer had any inhibitory effect on the HVA current. B, Temporal record of peak HVA current amplitude from the experiment in A. HVA current exhibits a continuous run-up with GDPβS. At early times after initiation of whole-cell recording, 10 μM carbachol is able to partially reduce HVA current (27 ± 6.2%; mean ± standard deviation; seven experiments). Inhibition by carbachol was blocked by atropine (1 μM; $n = 3$). C, Temporal record of peak HVA current amplitude is plotted for an experiment in which the pipette contained GTPγS. After run-down of >70% of the HVA current, 10 μM (+)-ACN still blocks >80% of the HVA current. Application of 1 μM ω-CgTx GVIA indicates that most of the (+)-ACN-blockable current is N-type current.

Those steroids with a relative lack of effect on total HVA current allow the conclusion that relatively minor structural changes among steroids can substantially change the blocking effects of these steroids. Because DRG neurons allow somewhat easier separation of different current components, we compared the ability of 30 μM (+)-ACN and 30 μM (−)-ACN to block N-, Q/R-, and R-type current in DRG neurons. Similar to the results on total HVA current in hippocampal neurons, (+)-ACN is slightly more effective than (−)-ACN in blocking each of the individual current components. For N-type current, the fractional blockade by 30 μM (−)-ACN was 0.76 ± 0.09 (five experiments) of that elicited by 30 μM (+)-ACN. For combined Q/R-type currents, the fractional blocking effect of (−)-ACN was 0.61 ± 0.08 (four experiments) of that of (+)-ACN. For R-type current, the current blocked by (−)-ACN was 0.65 ± 0.08 (three experiments) of that blocked by (+)-ACN. This suggests that the relative sensitivity of each HVA current to (+)-ACN and (−)-ACN is similar and that blockade of total HVA current by the two enantiomers involves the same set of I_{Ca} subtypes. Furthermore, there is clear enantioselectivity in the effects of (+)-ACN. It is worth noting that assuming similarly shaped concentration-response curves for both (+)-ACN and (−)-ACN, a reduction in block in the range of 0.61–0.76 could correspond to shifts in IC_{50} values of as much as 3–8-fold.

Discussion

One of the key findings of this work is that (+)-ACN, an anesthetic steroid, completely and reversibly blocks Q-, N-, and R-type HVA I_{Ba} without affecting L- and P-type HVA current in two different cells, neonatal rat hippocampal neurons and acutely dissociated DRG neurons. Although N- and Q-type currents are somewhat more sensitive to (+)-ACN blockade than R-type current, the IC_{50} value for blockade in

TABLE 1
Blockade of Total HVA Current in Hippocampal Neurons
Ratios were determined from the amount of block by 30 μM compound normalized to effect of 30 μM (+)-ACN.

Compounds	Blockade	n	Relative Blocking Ratio by 30 μM Compound	Potentiation of I_{GABA}
	%			%
(+)-ACN	44 ± 9	15	1	1829[a]
(−)-ACN	30 ± 16	7	0.65 ± 0.21	209[b]
Pregnanolone	36 ± 5	6	0.85 ± .07	1023[a]
Alfaxalone	7 ± 2	5	0.17 ± 0.07	
Pregnenolone	5 ± 1	5	0.13 ± 0.05	
(+)-DHP	11 ± 7	4	0.24 ± 0.08	1111[a]
PS	−8 ± 4	4	−0.16 ± 0.07	

[a] Potentiation by 10 μM compound of response to 2 μM GABA (Han *et al.*, 1996).
[b] Previously unpublished (but see Wittmer *et al.*, 1996).

each case is in the range of ~3–30 μM (+)-ACN. The effects of (+)-ACN do not involve G protein-mediated inhibitory pathways, suggesting that inhibition may result from specific binding sites on Ca^{2+} channels. A number of other steroids were much less effective at reducing HVA current, indicative that compounds must meet specific structural requirements to produce blockade. This suggests that selective structural alterations of particular steroids may result in compounds with more specificity, greater potency, or both. Specific, reversible, small-molecular-weight blockers of HVA I_{Ca} subtypes would provide valuable aides in the investigation of the physiological roles of different central nervous system Ca^{2+} channel variants. Because the effects of (+)-ACN on I_{Ba} occur at concentrations higher than those that produce effects on $GABA_A$ receptors, the anesthetic effects of (+)-ACN (Wittmer *et al.*, 1996) are unlikely to involve any effects on HVA I_{Ca}.

The selectivity in block of HVA current components by (+)-ACN. Our assertion that (+)-ACN selectively blocks N-, Q-, and R-type currents over P- and L-type currents requires that different components of HVA current can be clearly distinguished. Separation of L- and N-type currents can be relatively easily accomplished with nifedipine and ω-CgTx GVIA. We observed no reduction in the blocking effects of (+)-ACN after blockade of P- and L-type currents (Figs. 2 and 3). The pharmacological effects of ω-Aga IVA and ω-CmTx MVIIC are more complicated. ω-Aga-IVA seems to inhibit both P- and Q-type current at higher concentrations (1 μM) (Randall and Tsien, 1995), whereas for sufficiently brief applications at ≤0.2 μM, the effects of ω-Aga IVA are thought to be primarily on P-type current (Mintz *et al.*, 1992; Randall and Tsien, 1995). Similar separations between ω-Aga IVA- and ω-CmTx MVIIC-sensitive currents have been reported in rat DRG neurons (Rusin and Moises, 1995) and in a number of rat central and peripheral neurons (McDonough *et al.*, 1996). However, Tottene *et al.* (1996), using rat cerebellar Purkinje cells, did not find a current distinguishable by ω-Aga IVA and ω-CmTX MVIIC. Our observations support the former view and provide additional evidence for the idea that P and Q components are pharmacologically distinguishable. Specifically, (+)-ACN has no effect on that current removed by brief treatments with 0.1–0.2 μM ω-Aga IVA (Fig. 2), whereas (+)-ACN does block current also blockable by ω-CmTx MVIIC. In the case of ω-CmTx MVIIC, its ability to inhibit N-, P-, and Q-type currents can complicate interpretation of its actions. Similar to previous work (McDonough *et al.*, 1996), we observed that a rapid and reversible block of I_{Ba} produced by ω-CmTx MVIIC corresponded to the ω-CgTx GVIA-sensitive N-type current. We did not evaluate whether ω-CmTx MVIIC could slowly block P-type current, but we did observe that a slowly blocked, ω-CmTx MVIIC-sensitive current persisted after blockade by 0.1 μM ω-Aga-IVA (Figs. 2 and 5). Overlap of the ω-CmTx MVIIC-sensitive current with current blocked by (+)-ACN strongly supports the view that part of the action of (+)-ACN involves blockade of a Q-type current (Fig. 5).

The sensitivity of both P- and Q-type currents to ω-Aga IVA and ω-CmTx MVIIC has led to the suggestion that there may be some similarity in molecular components of these channels (Stea *et al.*, 1994; De Waard *et al.*, 1996). One proposal is that differences between P- and Q-type currents may arise from the β subunits that are coassembled with the α1A subunits (Moreno *et al.*, 1997). The current results suggest that (+)-ACN exhibits strong specificity in its ability to distinguish between Q- and P-type currents in both hippocampal and DRG neurons. This suggests that Q- and P-type channels must contain at least some different molecular components.

(+)-ACN also has significant blocking effects on presumed R-type current. After prior blockade of all other current components, an inhibitory action of (+)-ACN on the remaining current persisted (Figs. 4 and 5). This assertion is tempered by the fact that some of the residual current may reflect unblocked channels of other types (e.g., some Q-type current). R-type current is now suspected to arise from α1E subunits (Randall and Tsien, 1997). In support of the idea that R-type current is blocked by (+)-ACN, current arising from α1E Ca^{2+} channel subunits is also blocked by (+)-ACN with a concentration dependence similar to the block of R-type current observed here (Nakashima *et al.*, in preparation).

Ca^{2+} channels as possible targets of anesthetics. (+)-ACN is a potent anesthetic in both tadpole and mouse anesthesia assays (Wittmer *et al.*, 1996). As with other anesthetics (Jones *et al.*, 1992; Hara *et al.*, 1994), the ability of (+)-ACN to potentiate $GABA_A$-activated currents probably accounts for its anesthetic effects. The potential role, if any, of I_{Ca} inhibition in the actions of anesthetics remains unclear. Both L- and T-type I_{Ca} have been reported to be somewhat affected by anesthetics at concentrations that may occur clinically (Herrington *et al.*, 1991; Study, 1994; Todorovic and Lingle, 1998), whereas several general anesthetics were reported to be ineffective at blocking P-type current in rat Purkinje neurons (Hall *et al.*, 1994). Thus, different types of Ca^{2+} channels may exhibit differential sensitivities to various anesthetic compounds. However, in most cases, the effects of anesthetics on I_{Ca} are probably secondary compared with the ability of the same compounds to potentiate $GABA_A$-mediated currents (Franks and Lieb, 1994). For (+)-ACN, its relative lack of effect on any HVA current at concentrations of ≤1 μM argues that the behavioral effects of (+)-ACN do not involve effects on HVA I_{Ca}. However, we cannot exclude the possibility that for other steroids, Ca^{2+} channel inhibition may occur at concentrations producing anesthesia.

Steroids as potential probes of Ca^{2+} channels and the mechanism of (+)-ACN action. (+)-ACN does not strongly distinguish among N-, Q-, and R-type currents, although N-type current seems to be the most sensitive to (+)-ACN. Yet, (+)-ACN seems to be somewhat unique in its apparently absolute specificity between Q- and P-types of I_{Ca}. We consider this a useful starting point for an attempt to identify reversible, small-molecular-weight blockers of specific subtypes of Ca^{2+} channels.

A comparison of the relative effectiveness of various steroids on inhibition of total HVA I_{Ca} with ability to potentiate $GABA_A$ responses (Table 1) indicates a different rank order of potency for the two effects. This comparison must be taken with some caution because, except for (+)-ACN and (−)-ACN, we do not have any information about the specificity of action of other Ca^{2+} channel-blocking steroids among different HVA current components. However, because some compounds have essentially no effect on Ca^{2+} channels, we can conclude that the structural requirements of the site involved in steroid inhibition of some Ca^{2+} channels must be different from that on $GABA_A$ channels.

It has been reported that inhibition of some components of I_{Ca} in guinea pig hippocampal neurons by pregnenolone and PS involves a G protein-mediated pathway (ffrench-Mullen *et al.*, 1994). Because in the cultures of rat hippocampal neurons used here, we have been unable to find any blocking effects of pregnenolone or PS, we believe that these earlier reports are unrelated to the blocking effects of (+)-ACN. Furthermore, the current results argue strongly that the blocking effects of (+)-ACN do not involve a G protein-mediated pathway. Interference of G protein-mediated pathways with either GTPγS or GDPβS had no effect on the ability of (+)-ACN to inhibit HVA current in DRG neurons. Although we cannot rigorously exclude the possibility that Q- and R-type current inhibition might involve G protein pathways, we consider this unlikely for two reasons. First, inhibition by (+)-ACN seems to approach 100% for all current components. G protein-mediated inhibition typically results in only partial inhibition of an I_{Ca} (e.g., Shapiro and Hille, 1993; Viana and Hille, 1996). Second, the reproducibility and reliability of the inhibition by (+)-ACN contrast with the often-desensitizing nature of G protein-mediated inhibition of I_{Ca} (Ikeda and Schofield, 1989; Shapiro and Hille, 1993).

In summary, (+)-ACN blocks N-, Q-, and R-type Ca^{2+} channels with essentially no effects on P- and L-type channels. Inhibition of Ca^{2+} channels by (+)-ACN seems to involve direct interaction of steroids with the Ca^{2+} channels. The structural requirements for steroid blockade of Ca^{2+} channels differ from the requirements for effects on $GABA_A$ receptors. Thus, modifications of steroid structures may be a fruitful approach to the development of new small-molecular-weight, potent, specific, and reversible blockers of HVA I_{Ca}.

Acknowledgments

We are grateful to the Zorumski lab (Washington University School of Medicine, St. Louis, MO) for preparation of hippocampal microislands.

References

De Waard 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.

Eckstein F, Cassel D, Levkovitz H, Lowe M, and Selinger Z (1979) Guanosine 5′-*O*-(2-thiodiphosphate): an inhibitor of adenylate cyclase stimulation by guanine nucleotides and fluoride ions. *J Biol Chem* **254:**9829–9834.

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.

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.

Franks NP and Lieb WR (1994) Molecular and cellular mechanisms of general anaesthesia. *Nature (Lond)* **367:**607–614.

Gross RA and MacDonald RL (1988) Differential actions of pentobarbitone on calcium current components of mouse sensory neurones in culture. *J Physiol (Lond)* **405:**187–203.

Hall AC, Lieb WR, and Franks NR (1994) Insensitivity of P-type calcium channels to inhalational and intravenous general anesthetics. *Anesthesiology* **81:**117–123.

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. *Pflueg Arch Eur J Physiol* **391:**85–100.

Han M, Zorumski CF, and Covey DF (1996) Neurosteroid analogues. 4. The effect of methyl substitution at the C-5 and C-10 positions of neurosteroids on electrophysiological activity at $GABA_A$ receptors. *J Med Chem* **39:**4218–4232.

Hara M, Kai Y, and Ikemoto Y (1994) Enhancement by propofol of the $GABA_A$ response in dissociated hippocampal pyramidal neurons of the rat. *Anesthesiology* **81:**922–994.

Harrison NL and Simmonds MA (1984) Modulation of the GABA receptor complex by a steroid anaesthetic. *Brain Res* **323:**287–292.

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.

Holz GG, Rane SG, Dunlap K (1986) GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. *Nature (Lond)* **329:**670–672.

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.

Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G protein $\beta\gamma$ subunits. *Nature (Lond)* **380:**255–258.

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

Ishibashi H, Rhee JS, and Akaike N (1995) Regional differences of high voltage-activated Ca^{2+} channels in rat central nervous system neurones. *Neuroreport* **6:**1621–1624.

Jones MV, Brooks PA, and Harrison NL (1992) Enhancement of GABA-activated Cl currents in cultured rat hippocampal neurones by three volatile anesthetics. *J Physiol (Lond)* **449:**279–293.

Kavaliers M and Wiebe JP (1987) Analgesic effects of the progesterone metabolite, 3α-hydroxy-5α-pregnan-20-one, and possible modes of action in mice. *Brain Res* **415:**393–398.

McDonough SI, Swartz KJ, Mintz IM, Boland LM, and Bean BP (1996) Inhibition of calcium channels in rat central and peripheral neurons by ω-conotoxin MVIIC. *J Neurosci* **16:**2612–2623.

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

Mintz IM, Adams ME, and Bean BP (1992) P-type calcium channels in rat central and peripheral neurons. *Neuron* **9:**85–95.

Mintz IM and Bean BP (1993) GABA-B receptor inhibition of P-type Ca^{2+} channels in central neurons. *Neuron* **10:**889–898. [rf]Olcese R, Usai C, Maestrone E, and Nobile M (1994) The general anesthetic propofol inhibits transmembrane calcium current in chick sensory neurons. *Anesth Analg* **78:**955–960.

Moreno H, Rudy B, Llinas R (1997) *beta* Subunits influence the biophysical and pharmacological differences between P- and Q-type calcium currents expressed in a mammalian cell line. *Proc Natl Acad Sci USA* **94:**14042–14047.

Randall A and Tsien RW (1995) Pharmacological dissection of multiple types of Ca^{2+} channel currents in rat cerebellar granule neurons. *J Neurosci* **15:**2995–3012.

Randall A and Tsien RW (1997) Contrasting biophysical and pharmacological properties of T-type and R-type calcium channels. *Neuropharmacology* **36:**879–893. [rf]Rusin KI and Moises HC (1995) μ-opioid receptor activation reduces multiple components of high-threshold calcium current in rat sensory neurons. *J Neurosci* **15:**4315–4327.

Rodgers-Neame NT, Covey DF, Hu Y, Isenberg KE, and Zorumski CF (1992) Effects of a benz[*e*]indene on γ-aminobutyric acid-gated chloride currents in cultured postnatal hippocampal neurons. *Mol Pharmacol* **42:**952–957.

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

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

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.

Stea A, Tomlinson JW, Soong TW, Bourinet E, Dubel SJ, Vincent SR, and Snutch TP (1994) Localization and functional properties of a rat brain α1A calcium channel reflects similarities to neuronal Q- and P-type channels. *Proc Natl Acad Sci USA* **91:**10575–10580.

Study RE (1994) Isoflurane inhibits multiple voltage-gated calcium currents 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.

Tottene A, Moretti A, and Pietrobon D (1996) Functional diversity of P-type and R-type calcium channels in rat cerebellar neurons. *J Neurosci* **16:**6353–6363.

Viana F and Hille B (1996) Modulation of high voltage-activated calcium channels by somatostatin in acutely isolated rat amygdaloid neurons. *J Neurosci* **16:**6000–6011.

Wieland S, Belluzzi JD, Stein L, and Lan NC (1995) Comparative behavioral characterization of the neuroactive steroids 3 α-OH, 5 α-pregnan-20-one and 3 α-OH,5 β-pregnan-20-one in rodents. *Psychopharmacology* **118:**65–71.

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.

Send reprint requests to: Dr. Chris Lingle, Washington University School of Medicine, Dept. of Anesthesiology, Box 8054, St. Louis, MO 63110. E-mail: clingle@morpheus.wustl.edu