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Selectivity of Linopirdine (DuP 996), a Neurotransmitter Release Enhancer, in Blocking Voltage-Dependent and Calcium-Activated Potassium Currents in Hippocampal Neurons

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ABSTRACT

Linopirdine [DuP 996, 3,3-bis(4-pyridinylmethyl)-1-phenylindolin-2-one], a putative cognition enhancing drug, increases acetylcholine release in rat brain tissue and improves performance in animal models of learning and memory. The mechanism whereby linopirdine enhances acetylcholine release has been proposed to involve inhibition of the M-type K^+ current (I_M). Our study examines the selectivity of linopirdine for I_M by determining its effects on other ionic currents present in rat hippocampal CA_1 neurons using patch clamp techniques. Linopirdine was found to block voltage-gated, calcium-activated and leak K^+ currents in a dose-dependent manner. Of the seven currents measured, linopirdine was most selective for I_M with an IC_{50} of 2.4 ± 0.4 μM, followed by I_C (measured as a medium afterhyperpolarization tail current, I_{mAHP}) with an IC_{50} of 16.3 ± 2.4 μM. Both I_M and I_C were completely suppressed by linopirdine. At a concentration of 100 μM, linopirdine weakly inhibited the K^+ leak current, I_L, the transient outward current, I_A, the delayed rectifier, I_K, and the slow component of I_{AHP}, by 28 ± 8, 37 ± 10, 36 ± 9 and 52 ± 10 percent, respectively. The mixed Na^+/K^+ inward rectifying current, I_Q, was essentially unaffected by linopirdine (IC_{50} >300 μM). These results indicate that linopirdine selectively blocks I_M at concentrations ≤ 3 μM, the approximate EC_{50} for acetylcholine release enhancement. Inhibition of other voltage-gated and calcium-activated K^+ currents could also contribute to enhanced neurotransmitter release by linopirdine at intermediate (I_C) and high (I_L, I_A, I_K, I_{sAHP}) concentrations.

Linopirdine is a putative cognitive enhancing drug that increases stimulus-evoked release of a number of neurotransmitters, including ACh (Nickolson *et al.*, 1990; Zaczek *et al.*, 1995; Aiken *et al.*, 1996). Although the exact mechanism by which linopirdine enhances ACh release is unknown, Maciag *et al.* (1994) showed that its effects were insensitive to 4-aminopyridine, atropine and Na^+, Cl^- and Ca^{++} channel antagonists. In addition, they found no apparent role for cholinergic autoreceptors. TEA was found to have actions similar to linopirdine on ACh release suggesting the involvement of a K^+ channel in the action of linopirdine. Recently, Aiken *et al.* (1995) demonstrated that linopirdine reduced spike frequency adaptation and blocked I_M in rat hippocampal CA_1 neurons *in vitro*. Because the concentration-response curves for I_M block and ACh release have similar slopes and IC_{50}/EC_{50} values, it has been proposed that M-current block may represent the mechanism underlying linopirdine-induced neurotransmitter release enhancement (Aiken *et al.*, 1996).

Before ascribing pharmacological relevance to its M-current blocking activity, however, it is necessary to determine the effects of linopirdine on other K^+ channels. Many K^+ channel blockers have been shown to be non-selective. For example, TEA blocks I_C, I_K, I_M, $I_{K(IR)}$ and $I_{K(ATP)}$, 4-AP blocks I_A, I_D, some types of I_K and $I_{K(ATP)}$ and charybdotoxin blocks an intermediate-, as well as, the large-conductance calcium-activated K^+ channel (Cook and Quast, 1990; Halliwell, 1990). Thus, there may be other K^+ channels more sensitive than the M channel to the blocking action of linopirdine. In addition, a number of other potassium currents have been implicated in the control of neurotransmitter release. 4-AP and α-dendrotoxin, at concentrations that inhibit I_A, increase the release of glutamate from guinea-pig cerebrocortical synaptosomes (Tibbs *et al.*, 1989). Amoroso *et al.* (1990) demonstrated enhanced release of γ-aminobutyric acid by block of

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ABBREVIATIONS: I_M, M-current; I_A, transient K^+-current; I_K, delayed rectifier K^+ current; I_{mAHP}, medium calcium-activated K^+ current; I_C, TEA-sensitive, large conductance calcium-activated K^+ current; I_{sAHP}, slow calcium-activated K^+ current; I_L, leak potassium current; I_Q, slow inward rectifier Na^+/K^+ current; I_D, slowly inactivating, highly 4-aminopyridine-sensitive, delay K^+ current; $I_{K(ATP)}$, ATP-sensitive K^+ current; ACh, acetylcholine; TEA, tetraethylammonium; 4-AP, 4-aminopyridine; TTX, tetrodotoxin.

an adenosine triphosphate-sensitive K^+ channel in substantia nigra, and Robitaille *et al.* (1993) reported an increase in transmitter release at the neuromuscular junction produced by block of Ca^{++}-gated K^+ channels.

Our study was undertaken to determine the selectivity of linopirdine for I_M by determining its effects on the voltage-gated K^+ currents I_M, I_A and I_K, the afterhyperpolarization currents, I_{mAHP} and I_{sAHP}, the leak current, I_L, and the inward rectifier, I_Q, recorded from rat pyramidal CA_1 neurons in the hippocampal slice. Portions of this work have previously been published in a preliminary form (Schnee and Brown, 1995).

Methods

Studies in this report were carried out in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (Rockville, MD).

Tissue preparation. Pathogen-free male CD rats from Charles River (Wilmington, MA) weighing 30 to 80 g (15–25 days old) were anaesthetized with halothane. After decapitation, the brain was rapidly excised (<1 min) and submerged in an ice-cold oxygenated physiological solution. The brain was bisected and transverse slices containing the hippocampus were prepared on a Vibratome tissue slicer. Slices were transferred to a Perspex holding chamber filled with chilled saline and allowed to reach room temperature (23°C). For recording, slices were placed on a nylon mesh in a submersion-type chamber (Medical Systems, Greenvale, NY), pinned to a Sylgard base and perfused with an oxygenated physiological saline solution at room temperature at a rate of 3 ml min^{-1}. The physiological solution for both dissection and recording was (mM) NaCl (127.0), $NaHCO_3$ (26.0), KCl (3.0), $CaCl_2$ (2.5), NaH_2PO_4 (1.25), $MgSO_4$ (1.0) and glucose (10.0), gassed with 5% CO_2 in O_2 (pH 7.35). TTX (0.1 μM) or Cd^{++} (0.3 mM) were added to the perfusion solution to block Na^+ and Ca^{++} currents, respectively.

Electrophysiological recording. Microelectrodes were pulled from borosilicate glass (1.5 mm OD/1.0 mm ID; World Precision Instruments, Sarasota, FL) using a Sutter P-80/PC electrode puller (Sutter Instruments, Novato, CA). Electrode resistances were 2–2.5 Mohms when filled with intracellular solution. Tight-seal (1–5 Gohm) whole-cell voltage-clamp recordings, with access resistances of ≤ 20 Mohms, were obtained from neurons in the CA_1 pyramidal cell body layer using the "blind" patch technique. Current recordings were obtained by means of an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Signals were filtered at 5 KHz and recorded with pClamp software (version 6.0.1, Axon Instruments). Series resistance compensation was not used in order to minimize noise and "ringing" of the amplifier. In preliminary studies, little difference in current amplitudes was noted between the presence and absence of series resistance compensation when using 2 to 2.5 Mohm resistance electrodes. Except where stated, application time of drugs was approximately 20 min. Internal solutions for whole cell recording were (in mM) Kgluconate (140), KCl (10), HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) (10), EGTA (ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid) (10), $MgCl_2$ (2.0), $CaCl_2$ (1.0) and MgATP (2.0), pH adjusted to 7.4 with KOH; later experiments used KMethylsulfate (140) or KAspartate (130), KCl (10), HEPES (10), BAPTA (1,2-bis(2-aminophenoxy)ethane- N,N,N′,N′-tetraacetic acid) (10), K_2ATP (adenosine 5′-triphosphate, dipotassium salt) (5.0), $MgCl_2$ (2.0) and $CaCl_2$ (1.0), pH adjusted to 6.7 (to avoid rundown; Brown *et al.*, 1989; Cloues and Marrion, 1996) with KOH to record I_M and pH 7.3 to record other currents.

Drugs. Linopirdine (free base) was synthesized at the DuPont Merck Pharmaceutical Company (Wilmington, DE). A stock solution in 0.1 N HCl was prepared immediately before use and added to the superfusing solution. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Data analysis. All calculated data are expressed as mean ± S.E.M. In cumulative dose-response experiments on the effect of linopirdine on I_L amplitude over a range of voltage steps, a two-factor analysis of variance model (SuperANOVA, version 1.11, Abacus Concepts, Berkeley, CA) was used to test the hypothesis that mean control (pretreatment) values were unchanged with drug treatment. If there was evidence of a statistically significant treatment effect, Duncan's New Multiple Range test (SuperANOVA) was used to identify the significant dose effects. Statistical significance level was set at $P < .05$.

Results

Voltage-Gated Currents

I_M. M-current activates at potentials positive to −70 mV and is fully activated at −30 mV (Brown, 1988). To record I_M, hippocampal CA_1 neurons were stepped to −30 mV for 30 sec from a holding potential of −50 mV, repolarized by 20 mV for 2 sec and then stepped to −30 mV for 10 sec. Using this protocol, M-current deactivated in a mono-exponential manner during the 20 mV repolarization step. The amplitude of I_M was measured by fitting a single exponential function (Clampfit, Axon Instruments) to the outward deactivation tail current and extrapolating to the onset of the repolarization step. Each measure of I_M was the mean of nine repetitions of this protocol. Time constants for current deactivation from −30 mV were in the range of 110 to 220 msec, which were comparable to previously published reports (Brown, 1988). I_M in this preparation was sensitive to 50 μM carbachol and 1 mM Ba^{++} (figs. 1A and B) and was stable for >60 min after breaking into whole cell mode (amplitude was 106 ± 5, 104 ± 6 and 104 ± 9% of control after 20, 40 and 60 min, respectively).

A comparison of the I_M current relaxation before (control) and after a 20-min bath application of 3 μM linopirdine is illustrated in figure 1C. In this particular cell, linopirdine reduced the peak current by 64% without significantly affecting the rate of deactivation. In 11 neurons tested, linopirdine at 3 μM reduced I_M by 63 ± 4%. The time constant of I_M deactivation was unaffected by linopirdine (131 ± 7 msec in control, 151 ± 14 msec with 3 μM linopirdine). I_M block was only weakly reversible, even with extended wash (>60 min). Figure 1D shows the concentration response for I_M inhibition by linopirdine with a calculated IC_{50} of 2.4 ± 0.4 μM and a Hill slope of 1.9. Complete block of I_M by linopirdine was observed in three of five cells at 10 μM and two of two at 30 μM. Thus, the potency of linopirdine in blocking I_M in this preparation was comparable to that reported by Aiken *et al.* (1995) using single electrode voltage clamp in hippocampal CA_1 neurons and in cultured sympathetic cervical ganglia cells using the perforated patch technique (Lamas *et al.*, 1997).

I_A. The transient outward potassium current, I_A, observed in these studies was initially characterized by determining its electrophysiological and pharmacological properties. Steady-state inactivation was studied by applying, from a holding potential of −50 mV, 100 msec prepulses to potentials of −20 to −110 mV followed by a 300 msec voltage step to +50 mV. Steady state activation was examined by clamping to potentials between −40 and +50 mV from a −50 mV holding potential in 10 mV increments. These studies showed

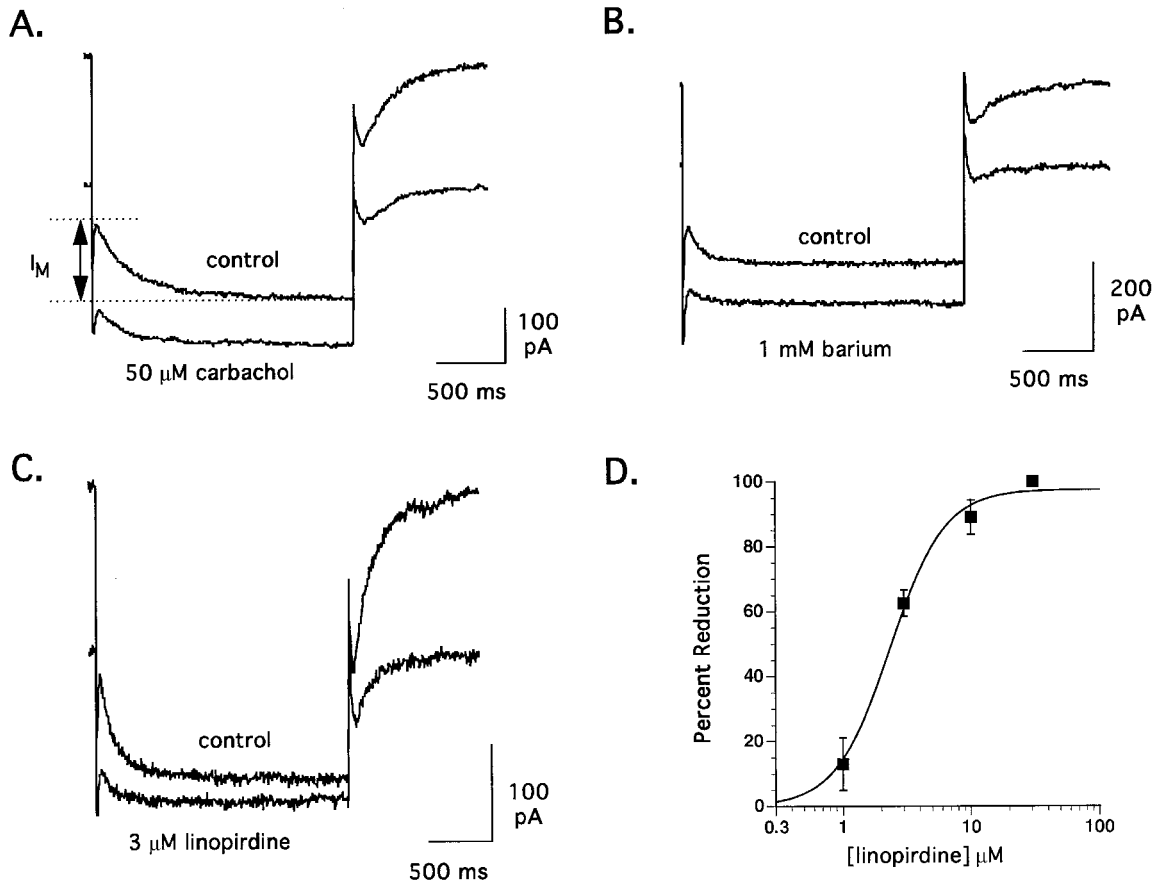

Fig. 1. Representative traces of the effect of 50 μM carbachol (A), 1 mM barium (B) and 3 μM linopirdine (C) on M-current recorded from the soma of a CA_1 neuron in a rat hippocampal slice. Each trace illustrates the deactivation tail current during a 2 sec voltage step from −30 to −50 mV; the amplitude of which is equivalent to the M-current. (D) Concentration-response curve for the inhibition of M-current by linopirdine (n = 5, 11, 5 and 2 at 1, 3, 10 and 30 μM linopirdine, respectively). Recordings were made in the presence of 0.1 μM TTX and 0.3 mM Cd^{++} using a patch pipette with an internal pH of 6.7.

that >90% of I_A inactivation was removed by prepulses to potentials more negative than −75 mV and that I_A activated positive to −30 mV (fig. 2A). These characteristics, along with the observed block of I_A by 4-AP (fig. 2B), are consistent with known properties of this current.

To isolate I_A for the examination of linopirdine effects, slices were pretreated with 10 mM TEA to reduce the large delayed rectifier component of outward current present in CA_1 neurons (seen in fig. 2B). In the presence of TEA, I_A amplitude was determined by measuring the difference between baseline current at the holding potential of −50 mV and the peak of the transient current component following step depolarization (fig. 2B). The effects of 10, 30 and 100 μM linopirdine on 1) the voltage dependence of steady state inactivation and activation, 2) peak current amplitude during activation (fig. 2C) and 3) rate of inactivation were determined. Linopirdine had no effect on either the voltage dependence of steady-state inactivation or the voltage dependence of activation at any concentration studied. Linopirdine did, however, significantly reduce peak I_A amplitude and tau inactivation (fig. 2D) by 37 ± 10 and 49 ± 3%, respectively, at 100 μM. These results indicate that the effects of linopirdine on I_A were weak in comparison to its block of I_M.

I_K. The delayed rectifier current, I_K, observed in these studies was evoked by 2000 msec depolarizing steps to +60 mV from a holding potential of −50 mV in 10 mV increments without a hyperpolarizing prepulse. I_K, measured as the steady-state current amplitude at the end of each depolarizing step (baseline values were 5416 ± 654 pA at +60 mV; n = 13), activated at potentials positive to −30 mV (fig. 3A) and only slowly inactivated during the command pulse (fig. 3B). These characteristics, along with the blocking effects of TEA (fig. 3B), are established properties of I_K.

For the examination of linopirdine effects, cells were recorded with an intracellular pipette solution that allowed the rundown of I_M within 5 min (pH = 7.3). Under these conditions, linopirdine, at 10 to 100 μM, reduced the amplitude of I_K in a dose-dependent manner (figs. 3C and D) with the concentration that inhibited I_K by 50% exceeding 100 μM. Linopirdine-induced block of I_K was voltage-independent as activation $V_{1/2}$ values were 14.4 ± 0.4, 15.0 ± 0.6, 15.3 ± 0.7 and 17.4 ± 0.7 mV under control and 10, 30 and 100 μM linopirdine conditions, respectively. In contrast to I_A, linopirdine had no apparent effect on the rate of I_K inactivation at any concentration studied (fig. 3C).

Afterhyperpolarization Currents

The outward tail current recorded from hippocampal CA_1 neurons after 10 mV incremental depolarizing steps from a holding potential of −50 mV to +60 mV in the absence of calcium channel blockers (figs. 4A and 6B) appeared to be a composite of two currents which could be differentiated ki-

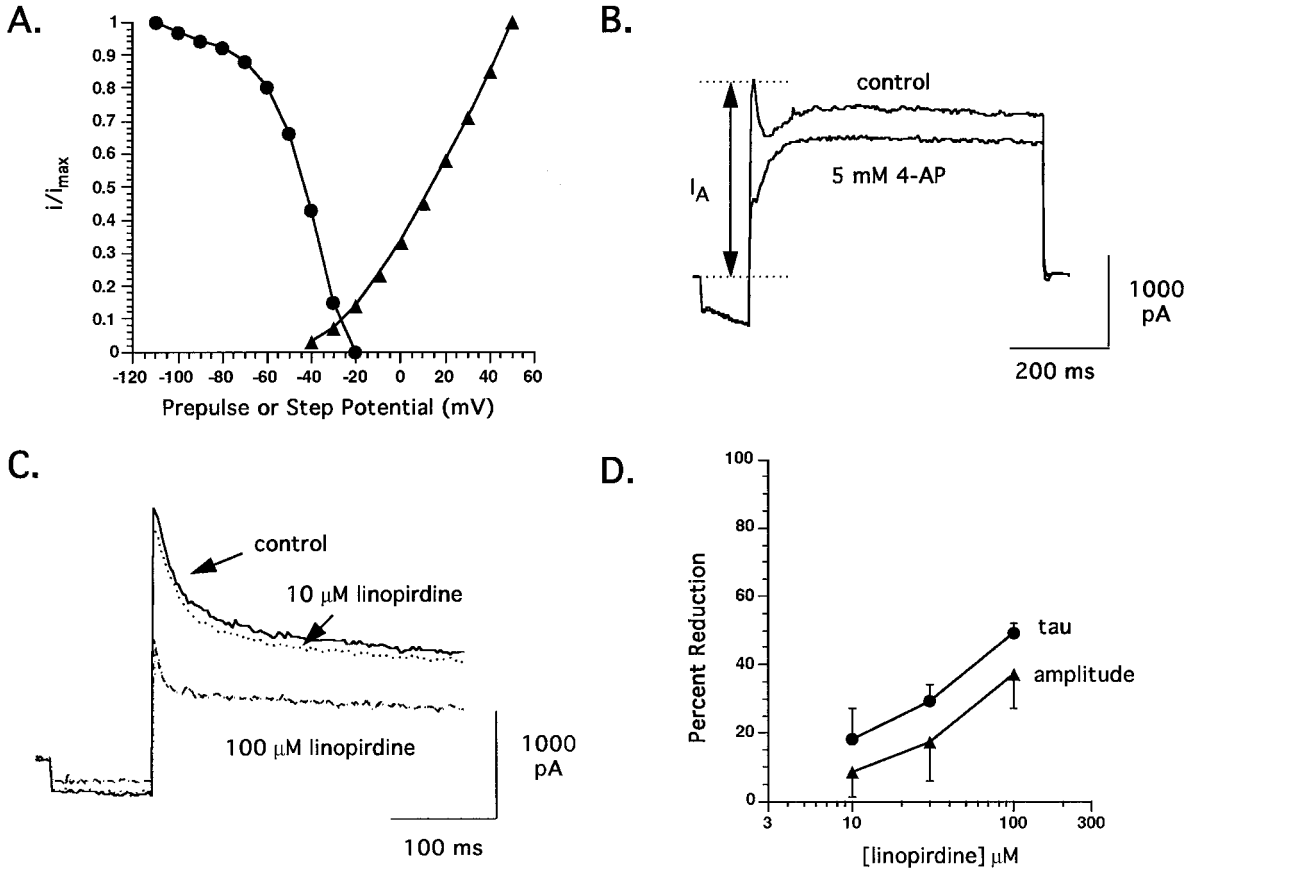

Fig. 2. A, Mean steady-state inactivation and activation curves for the peak of the transient outward current, I_A, rat hippocampal CA_1 neurons (n = 6). B and C, Representative traces of the effect of 4-AP (5 mM) and linopirdine (10, 100 μM) on I_A in neurons held at −50 mV, clamped to −100 mV for 100 msec to remove inactivation, then stepped to +50 mV to activate I_A. D, Concentration-response curve for the inhibition of I_A amplitude and inactivation rate constant (τ) by linopirdine (n = 6, 5 and 5 at 10, 30 and 100 μM linopirdine, respectively). Recordings were made in the presence of 0.1 μM TTX, 10 mM TEA (except for B) using a patch pipette with an internal pH of 7.3.

netically (medium and slow) as well as pharmacologically. Voltage steps of 3.5 to 108 msec duration to 10 to +60 mV elicited tail currents which decayed in a biexponential manner (figs. 4, B, C and D). The first component was not well resolved under the experimental conditions used. Results of the second exponential fit (as performed by the Clampex portion of pCLAMP; mixed method) were used to measure I_{mAHP} parameters. Maximum I_{mAHP} amplitude (744 ± 129 pA; n = 7) with a deactivation rate constant of 33 ± 7 msec (n = 7) was evoked by a 108 msec step to +60 mV. The slowest tail current component (referred to as I_{sAHP}) was activated by long (1.5 sec) depolarizing steps to −30 mV and above (fig. 6A). Typically, I_{sAHP} peaked 3 to 5 sec after termination of the depolarizing step (at an amplitude of 228 ± 45 pA; n = 14) and deactivated over the next several seconds (fig. 6B and C). The effect of linopirdine on I_{mAHP} and I_{sAHP} was determined using a step duration and amplitude at or near maximum for each current component; *i.e.,* a step to +60 mV for 54 ms for I_{mAHP} and 1.5 sec for I_{sAHP}.

I_{mAHP}. To characterize I_{mAHP} pharmacologically, the effects of TEA and cobalt, inhibitors of hippocampal I_C, were evaluated. TEA (1.5 mM) was effective in reducing the amplitude of I_{mAHP} (by 57 ± 4.5%; n = 6) (fig. 5A) and had no effect on I_{sAHP}. Cobalt (2 mM) also reduced I_{mAHP} amplitude (72%, n = 2), whereas cholinergic agonists (carbachol and muscarine, 5–50 μM) and norepinephrine (3–10 μM) had no consistent effect on I_{mAHP}. Because I_{mAHP} was blocked by TEA and cobalt, not blocked by carbachol and had a deactivation time constant of approximately 30 msec, it was assumed that I_{mAHP} is largely comprised of what is commonly referred to as I_C (Storm, 1990).

Linopirdine also inhibited I_{mAHP} (fig. 5B and C). At 3 μM, a concentration more than the IC_{50} for inhibition of I_M, linopirdine had no significant effect on I_{mAHP}. At 10 to 30 μM linopirdine, however, I_{mAHP} was significantly depressed and essentially completely blocked at 100 μM. Accordingly, the IC_{50} for linopirdine-induced inhibition of I_{mAHP} was 16.3 ± 2.4 μM (fig. 5C). As observed for I_M, the effect of linopirdine on I_{mAHP} was not readily reversible. However, this was not due to rundown of the current since, in control experiments, the amplitude of I_{mAHP} remained stable over the time period of linopirdine exposure (122 ± 10% of control at 60 min). In addition to I_M, the amplitude of I_{mAHP} is the only K^+ current in our study to be completely suppressed by linopirdine. The deactivation rate constant of I_{mAHP} was not significantly affected by linopirdine (data not shown).

I_{sAHP}. The current underlying the slow afterhyperpolarization in hippocampal pyramidal neurons can be blocked by several neurotransmitters, including norepinephrine and acetylcholine (Storm, 1990). To verify the identity of I_{sAHP} as the current commonly referred to in the literature as I_{AHP}, the effects of norepinephrine, muscarine and TEA were evaluated. In agreement with published reports, 10 μM norepinephrine (fig. 6B) and 10 μM muscarine markedly attenu-

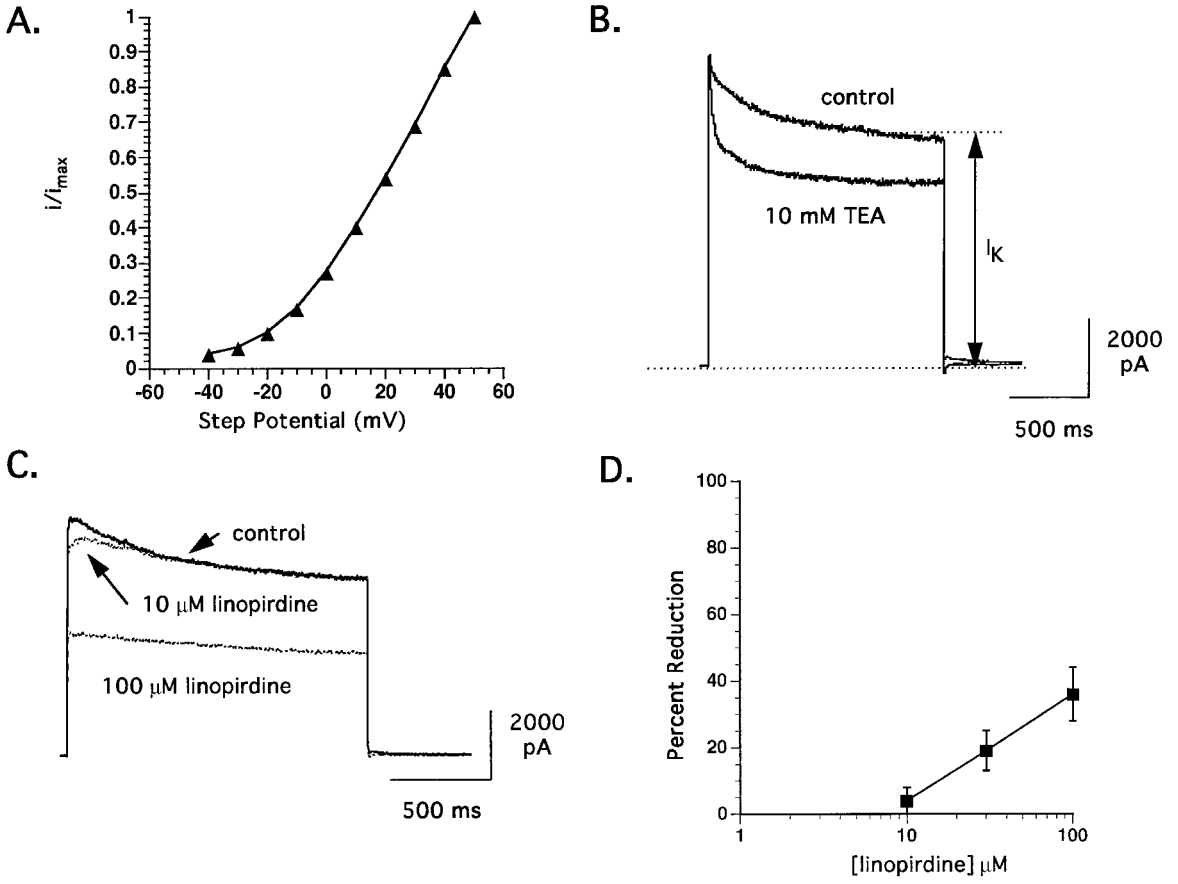

Fig. 3. A, Mean steady-state activation curve for the peak of the delayed rectifier outward current, I_K, rat hippocampal CA_1 neurons. B and C, Representative traces of the effect of TEA (10 mM) and linopirdine (10, 100 μM) on I_K in neurons held at −50 mV and stepped to +50 mV to activate I_K. D, Concentration-response curve for the inhibition of I_K amplitude by linopirdine (n = 9, 7 and 5 at 10, 30 and 100 μM linopirdine, respectively) Recordings were made in the presence of 0.1 μM TTX using a patch pipette with an internal pH of 7.3.

ated I_{sAHP}, whereas 1.5 mM TEA had no effect on I_{sAHP} in the same preparations in which it blocked I_{mAHP} by an average of 57 percent (data not shown).

Linopirdine exerted a concentration-dependent inhibition of the amplitude of I_{sAHP} while having little or no effect on the rate of deactivation (fig. 6C and D). Its effects on I_{sAHP} were, however, weak and similar to those on I_A and I_K in that a concentration of 30 μM was required to inhibit I_{sAHP} by approximately 50%, with no additional effect at 100 μM. These results indicate that, of the two components of depolarization-induced outward tail currents measured, linopirdine most potently blocked I_{mAHP}.

Inward Rectifier and Leak Current

I_Q. I_Q, a mixed Na^+/K^+ current, was recorded in CA_1 neurons in response to 10 mV incremental hyperpolarizing voltage steps from a holding potential of −30 mV (fig. 7A). I_Q slowly activates (τ = 241 ± 7.5 msec at −100 mV) at potentials negative to −60 mV, is noninactivating and is sensitive to 5 mM cesium (fig. 7B). Linopirdine, at 30 μM, had no effect on I_Q (fig. 7C) and, at concentrations as high as 300 μM, only slightly reduced I_Q (by 26%). Thus, the inwardly rectifying current examined in this study was even less sensitive to linopirdine than were the weakly inhibited I_A, I_K and I_{sAHP}.

I_L. I_L, a leak potassium current, was measured in CA_1 neurons as the instantaneous current component evoked by the voltage protocol utilized to activate I_Q (fig. 7C). In agreement with Benson *et al.* (1988), the instantaneous current was nonrectifying over the range of −40 to −100 mV (fig. 7D). In four cells, linopirdine induced a small, concentration-dependent reduction of I_L which was statistically significant at both 30 and 100 μM. Under control conditions, a voltage step from −30 to −100 mV induced an instantaneous current of −892 ± 108 pA which was reduced by 9 ± 5, 17 ± 8 and 28 ± 8% in the presence of 10, 30 and 100 μM linopirdine, respectively. These results indicate that, like I_A, I_K, I_{sAHP} and I_Q, I_L is weakly inhibited by linopirdine.

Discussion

Our objective was to determine the selectivity of linopirdine for I_M by measuring its effects on other voltage-dependent and calcium-activated K^+ currents. The order of sensitivity to linopirdine among the seven currents recorded from hippocampal CA_1 neurons was $I_M > I_{mAHP} \gg I_K = I_{sAHP} > I_L = I_A \gg I_Q$. Thus, linopirdine showed approximately seven times more selectivity for I_M than the next most sensitive current, I_{mAHP}, and a more than 50-fold selectivity for I_M over the other five measured currents. In addition, I_M and I_{mAHP} were the only K^+ currents that could be completely blocked by linopirdine.

The inhibition of I_M by linopirdine observed in our study confirms previous reports on its effects in rat hippocampal CA_1 neurons (Aiken *et al.*, 1995) and rat superior cervical

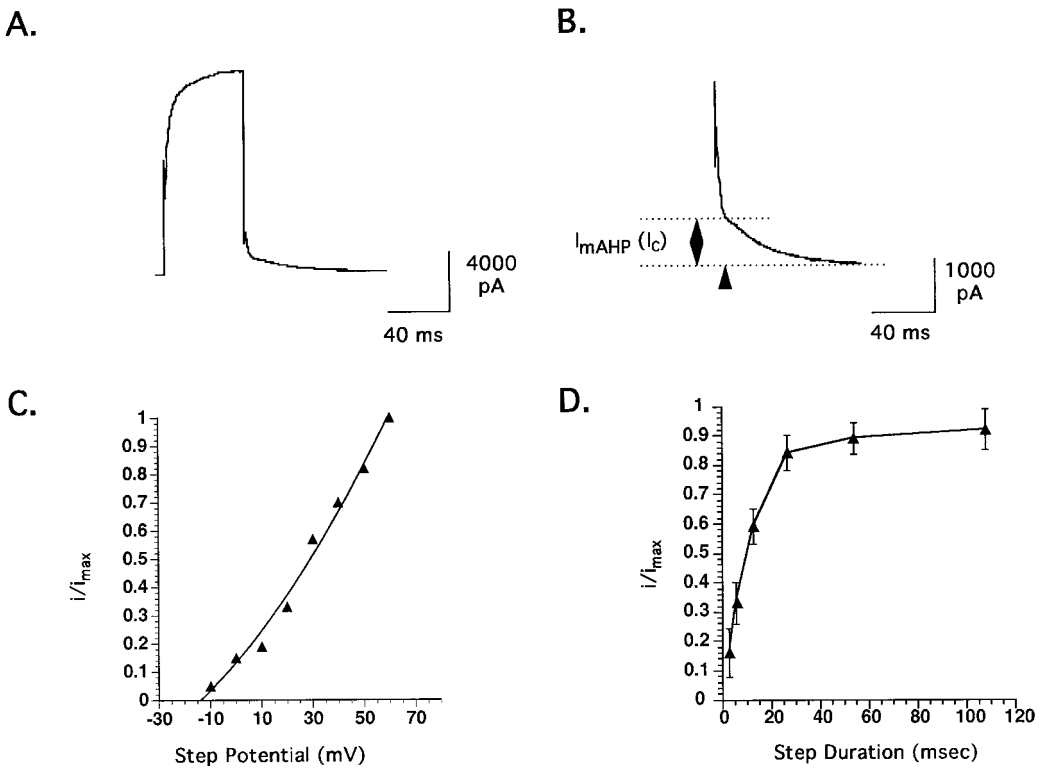

Fig. 4. A, Representative response of a rat hippocampal CA_1 neuron held at −50 mV, stepped to + 50 mV for 54 msec and returned to −50 mV. Upon repolarization to −50 mV, a biexponentially decaying tail current (an expansion of which is shown in B) can be visualized. The amplitude of the second component, measured at the time indicated by the solid triangle, was recorded as I_{mAHP}/I_C. The effect of step potential and duration of the preceding depolarizing pulse on the mean activation of I_{mAHP}/I_C (n = 8) is shown in C and D, respectively. Recordings were made in the presence of 0.1 μM TTX using a patch pipette with an internal pH of 7.3.

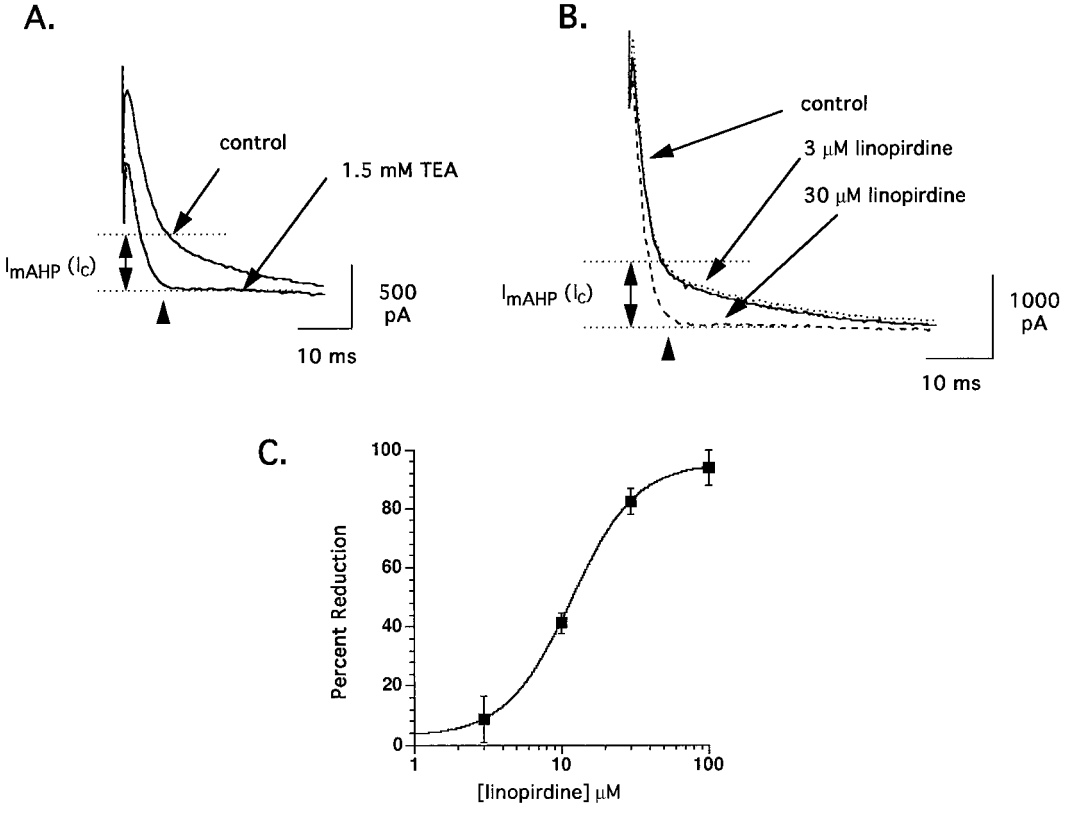

Fig. 5. A and B, Representative traces of the effect of TEA (1.5 mM) and linopirdine (3, 30 μM) on I_{mAHP}/I_C (measured as described in the legend to fig. 4) in neurons held at −50 mV, stepped to + 50 mV for 54 msec and returned to −50 mV. C, Concentration-response curve for the inhibition of I_{mAHP} by linopirdine after a 54 msec depolarization to +50 mV. (n values for I_{mAHP} were 5, 8, 8, 5, respectively, at 3, 10, 30 and 100 μM linopirdine.) Recordings were made in the presence of 0.1 μM TTX using a patch pipette with an internal pH of 7.3.

ganglia (Lamas *et al.*, 1997; Costa and Brown, 1997). The IC_{50} of 2.4 μM determined in this study agrees well with reported values in the range of 3.4 to 8.5 μM. In addition, in all four studies, linopirdine, in a concentration range of 30 to 100 μM, inhibited I_M by 100%. This, together with the lack of effect of internal GTPγS, GDPβS or BAPTA on I_M inhibition (Costa and Brown, 1997) and the block of M channels in outside-out membrane patches (Lamas *et al.*, 1997), strongly indicate that linopirdine is a direct M channel blocker as opposed to working through a G-protein- or calcium-coupled second messenger system. The ability of linopirdine to block M channels is, as previously discussed (Aiken *et al.*, 1995),

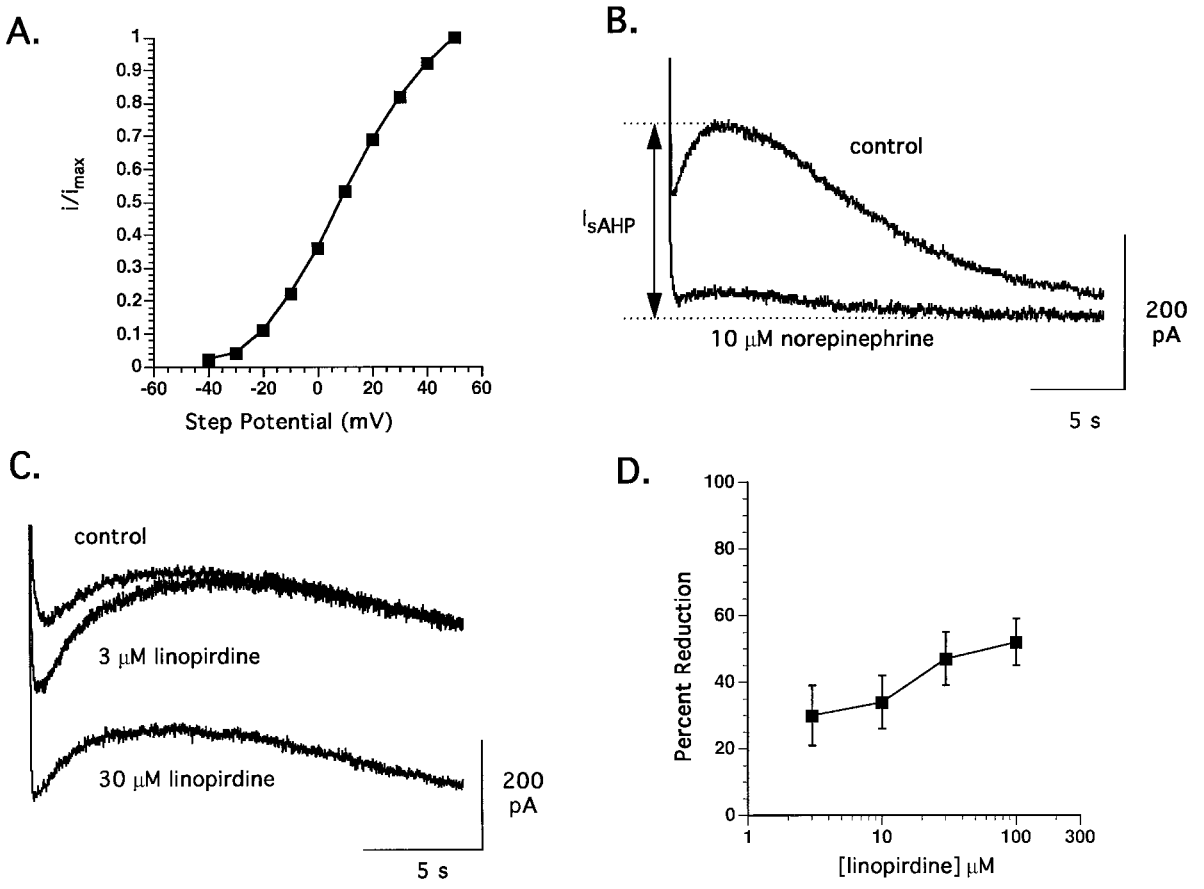

Fig. 6. A, Mean steady-state activation curve for the peak of the slow afterhyperpolarization tail current, I_{sAHP}, which occurs in rat hippocampal CA_1 neurons after a 1.5-sec depolarization step from a holding potential of −50 mV ($n = 8$). B and C, Representative traces of the effect of norepinephrine (10 μM) and linopirdine (3, 30 μM) on the slow afterhyperpolarization tail current. D, Concentration-response curve for the inhibition of I_{sAHP} by linopirdine ($n = 12$, 13, 11 and 7 at 3, 10, 30 and 100 μM linopirdine, respectively). Recordings were made in the presence of 0.1 μM TTX using a patch pipette with an internal pH of 7.3.

likely to account for its voltage-dependent depolarization of resting membrane potential and reduction of spike frequency adaptation.

Given the putative role of I_C in mediating spike repolarization (Storm, 1990), the block of I_{mAHP} (I_C) observed in the present study by linopirdine may account for its effects on action potential duration in hippocampal CA_1 neurons. In studies performed at room temperature, a single concentration of linopirdine (10 μM) had no effect on action potential duration (Aiken *et al.*, 1995). Under these conditions, action potential duration was already prolonged relative to physiologic temperature and, based on the IC_{50} of 16.3 μM determined in this study, I_C inhibition was likely to be less than 50%. However, in earlier studies performed at 37°C using a concentration range of 5 to 100 μM (Lampe and Brown, 1991), linopirdine exerted a concentration-dependent prolongation of action potential duration, with small effects (20–30% increase) noted at 10 μM and a more than 100% increase seen at 30 μM. In addition, the weak inhibition of I_A and I_K observed in this study (and by Lamas *et al.*, 1997 in rat superior cervical ganglia) may also contribute to the prolongation of action potential duration exerted by ≥ 30 μM linopirdine.

In current clamp studies using sharp microelectrodes, we observed that 10 μM linopirdine had no significant effect on: 1) normal resting membrane potential, 2) the voltage sag during a prolonged hyperpolarizing pulse from resting potential (Lampe and Brown, 1991) and 3) the slow afterhyperpolarization after a train of action potentials evoked by a prolonged depolarizing pulse (Aiken *et al.*, 1995). Because these potentials can, at least in part, be accounted for by I_L, I_Q and the I_{sAHP}, respectively, our findings of a weak or absent effect of 10 μM linopirdine on these currents are in agreement with the current clamp results. At ≥30 μM, we have seen a depolarization of normal resting potential induced by linopirdine (B.J. Lampe, P.A. Murphy and B.S. Brown, unpublished observation) which may be due to its small but significant inhibition of I_L.

Using cultured rat sympathetic ganglia, Lamas *et al.* (1997) also studied the effects of linopirdine on a variety of ionic currents. In general, there is good agreement between their results and those of our study in that linopirdine potently inhibited I_M, weakly inhibited I_A and I_K and, at 10 μM, had no significant effect on I_{AHP} or I_Q. One notable difference between the two studies, however, is the effect of linopirdine on I_C. In sympathetic ganglia, 10 μM linopirdine had no effect on I_C whereas in hippocampal CA_1 neurons, I_{mAHP}/I_C was inhibited at an IC_{50} of 16.3 μM and was one of two currents completely suppressed by linopirdine. The difference in the pharmacology of I_C between ganglia and CA_1 neurons may represent a difference in the expression of BK channel subtypes, because the

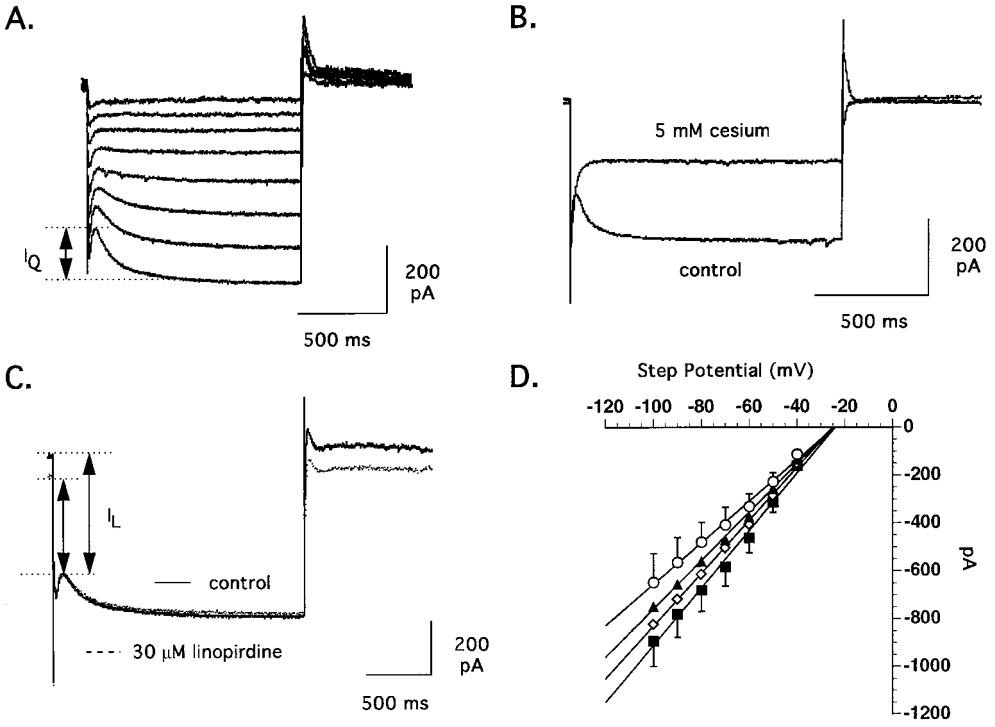

Fig. 7. A, Representative traces of the voltage-dependent activation of I_Q in rat hippocampal CA_1 neurons upon incremental 10 mV hyperpolarization steps to −100 mV from a holding potential of −30 mV. B and C, Effect of cesium (5 mM) or linopirdine (30 μM) on I_Q and I_L in neurons stepped to −100 from −30 mV. D, Concentration-dependent effect of linopirdine on I_L from −40 to −100 mV. ■ Baseline, ◇ 10 μM, ▲ 30 μM and ○ 100 μM linopirdine. For the purpose of clarity, standard error bars were not included on the 10 and 30 μM linopirdine lines. Recordings were made in the presence of 0.1 μM TTX using a patch pipette with an internal pH of 7.3.

activation of BK channels is believed to correspond to the I_C macroscopic current (Sah, 1996). A similar explanation has been proposed to account for the well known difference in the pharmacology of the small-conductance, calcium-activated K^+ current in sympathetic ganglia and hippocampus (Lancaster and Adams, 1986; Storm, 1989; Sah, 1996) where the ganglionic current is apamin-sensitive but the hippocampal current is apamin-insensitive. This pharmacological difference may be related to the expression of different SK channel subtypes in the two tissues (Kohler *et al.*, 1996).Similarly, because there are at least two major subtypes of BK channels (for review, see Gribkoff *et al.*, 1997), a difference in BK expression between sympathetic ganglia and hippocampal CA_1 neurons could explain the apparent difference in sensitivity of I_C to linopirdine in the two cell types.

Previous studies into the mechanism whereby linopirdine enhances neurotransmitter release indicated an inhibition of M-current as the most likely site of action (Aiken *et al.*, 1995, 1996). Our results support this conclusion and, in addition, suggest that the inhibition of I_C may also play an important role in the action of linopirdine because there was only a 7-fold separation between the IC_{50} values for the two currents. Accordingly, both an inhibition of I_C/BK channels and the resultant action potential broadening have been associated with enhancement of transmitter release (Robitaille *et al.*, 1993; Jackson *et al.*, 1991). Furthermore, although the mechanism of M-current block has been studied in some detail, with results indicating a direct channel interaction (Lamas *et al.*, 1997; Costa and Brown, 1998), similar studies have not been performed with BK channels. However, because most, if not all, known activators and blockers of BK channels are direct channel blockers (Gribkoff *et al.*, 1997), the same may also be true of linopirdine.

We have shown that linopirdine is a selective blocker of M-current at concentrations below 10 μM in hippocampal CA_1 neurons. At higher concentrations, it will first block I_C, then I_K, I_{sAHP}, I_L, I_A and I_Q. This progression of ion channel effects, as well as its interaction with peripheral ligand-gated channels (Lamas *et al.*, 1997), must be considered when interpreting the functional and toxicological properties of linopirdine.

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