

# Methyl-Laudanosine: A New Pharmacological Tool to Investigate the Function of Small-Conductance $\text{Ca}^{2+}$ -Activated $\text{K}^+$ Channels

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## ABSTRACT

Small-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (SK channels) underlie the prolonged postspike afterhyperpolarization (AHP) observed in many central neurons and play an important role in modulating neuronal activity. However, a lack of specific and reversible blockers of these channels hampers their study in various experimental conditions. Because previous work has shown that bicuculline salts block these channels, we examined whether related alkaloids, namely laudanosine quaternary derivatives, would produce similar effects. Intracellular recordings were performed on rat midbrain dopaminergic neurons and hippocampus CA1 pyramidal cells. Binding experiments were performed on rat cerebral cortex membranes. Laudanosine, methyl-laudanosine, and ethyl-laudanosine blocked the apamin-sensitive AHP of dopaminergic neurons with mean  $\text{IC}_{50}$  values of 152, 15, and 47  $\mu\text{M}$ , respectively. The benzyl and butyl derivatives were less potent. Methyl-laudanosine had no

effect on the  $\text{I}_h$  current, action potential parameters, or membrane resistance of dopaminergic cells, or on the decrease in input resistance induced by muscimol, indicating a lack of antagonism at  $\text{GABA}_A$  receptors. Interestingly, 100  $\mu\text{M}$  methyl-laudanosine induced a significant increase in spiking frequency of dopaminergic neurons but not of CA1 pyramidal cells, suggesting the possibility of regional selectivity. Binding experiments on laudanosine derivatives were in good agreement with electrophysiological data. Moreover, methyl-laudanosine has no affinity for voltage-gated potassium channels, and its affinity for SK channels ( $\text{IC}_{50}$  4  $\mu\text{M}$ ) is superior to its affinity for muscarinic ( $\text{IC}_{50}$  114  $\mu\text{M}$ ) and neuronal nicotinic ( $\text{IC}_{50} \geq 367 \mu\text{M}$ ) receptors. Methyl-laudanosine may be a valuable pharmacological tool to investigate the role of SK channels in various experimental models.

Other than neurotransmitter receptors and transporters, ion channels constitute an attractive target to develop new drugs that will be active on the central nervous system. Currently, the only ion channel that is well established as a central nervous system target is the voltage-gated  $\text{Na}^+$  channel, which is blocked by antiepileptic drugs such as phenytoin, carbamazepine, and lamotrigine (McNamara, 1996).

$\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels play a fundamental role in the control of the firing frequency and pattern of neurons (for reviews, see Sah, 1996; Vergara et al., 1998). Within this class of  $\text{K}^+$  channels, small-conductance voltage-insensitive  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (SK channels) underlie the prolonged postspike afterhyperpolarization (AHP) that is ob-

served in many central neurons. Three closely related SK-channel subtypes, SK1, SK2, and SK3, have been cloned and are characterized by different sensitivities to apamin (Köhler et al., 1996; Strøbæk et al., 2000). These subtypes are differentially expressed in the brain (Stocker and Pedarzani, 2000). Moreover, the currents underlying AHP have been classified into two groups on the basis of their kinetic and pharmacological properties.  $\text{I}_{\text{AHP}}$ , the current underlying the medium AHP, is present in most excitable cells; it is sensitive to the bee venom toxin apamin, and its activation has been reported to control firing frequency in tonically spiking neurons.  $\text{sI}_{\text{AHP}}$ , the current underlying the slow AHP, is present in a few cell types only; it is apamin-insensitive but can be modulated by various neurotransmitters, and its activation is responsible for late-spike frequency adaptation (Sah, 1996; Vergara et al., 1998).

Evidence suggests that SK-channel modulation may be interesting in a range of central nervous system disorders,

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**ABBREVIATIONS:** SK channels, small-conductance voltage-insensitive  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels; AHP, afterhyperpolarization; ZD7288, 4-(N-ethyl-N-phenylamino)-1,2-dimethyl-6-(methylamino)-pyrimidinium chloride; SR95531, 2-[carboxy-3'-propyl]-3-amino-6-paramethoxy-phenyl-pyridazinium bromide.

including Alzheimer's disease and schizophrenia. Indeed, apamin has been shown to improve some aspects of memory processing in animal models (Ikonen and Riekkinen, 1999; Fournier et al., 2001), and longer CAG repeats within the gene of the SK3 channel have been associated with schizophrenia in various ethnic groups (Chandy et al., 1998; Dror et al., 1999), although the latter observation is controversial (Antonarakis et al., 1999).

To validate SK channels (or one of their subtypes) as an interesting drug target, it is critical to develop adequate tools for studying their function. Apamin is not ideal because it induces a long-lasting block of SK channels; moreover, its peptidic nature precludes iontophoretic application during *in vivo* experiments. Several currently available nonpeptidic blockers of SK channels (e.g., (+)-tubocurarine, bicuculline salts, and gallamine) lack specificity, acting on other targets, including GABA<sub>A</sub>, nicotinic, or muscarinic receptors (Lee and el-Fakahany, 1991; Dunn et al., 1996; Seutin and Johnson, 1999; Strøbæk et al., 2000). Recently, novel nonpeptidic blockers of SK channels have been synthesized and evaluated. Among these bis-quinolinium cyclophanes, UCL1684 has been found to be the most potent compound, having an IC<sub>50</sub> value of 3 nM on AHP of rat superior cervical ganglion neurons (Campos Rosa et al., 2000). However, to our knowledge, a comprehensive screening of various receptors and channels is not available for these compounds, making it difficult to assess their degree of selectivity.

Given the structural analogy between bicuculline and laudanosine, a metabolite of the neuromuscular relaxant atracurium, we decided to evaluate the ability of laudanosine and its methyl, ethyl, butyl, and benzyl derivatives to selectively block SK channels. Both electrophysiological and binding experiments were performed. The electrophysiological study was done on rat midbrain dopaminergic neurons and hippocampus CA1 pyramidal cells. Midbrain dopaminergic neurons display a prominent apamin-sensitive AHP of medium duration, which appears to be mediated by SK3 channels (Shepard and Bunney, 1991; Wolfart et al., 2001). Hippocampus CA1 pyramidal cells present both a medium, apamin-sensitive and a slow, apamin-insensitive AHP (Stocker et al., 1999). SK1 and SK2 subunits are highly expressed in this region (Stocker and Pedarzani, 2000). Binding experiments on SK channels, voltage-gated potassium channels, and muscarinic and nicotinic receptors were performed on rat cerebral cortex membranes.

## Materials and Methods

**Electrophysiological Experiments.** The methods used were similar to those described previously (Seutin et al., 1997). Male Wistar rats (150–200 g) were used. They were housed and handled in accordance with guidelines of the National Institute of Health (National Institutes of Health Publication 85-23, 1985). Animals were anesthetized with chloral hydrate (400 mg/kg *i.p.*) and decapitated. The brain was excised quickly and placed in cold (~4°C) artificial cerebrospinal fluid of the following composition: 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgCl<sub>2</sub>, 2.4 mM CaCl<sub>2</sub>, 11 mM glucose, and 18 mM NaHCO<sub>3</sub>, saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4). A block of tissue containing the midbrain or the hippocampus was cut into horizontal or transverse slices, respectively (thickness 350 µm), in a Vibratome (Lancer, St. Louis, MO). The slice containing the region of interest was placed on a nylon mesh in a recording chamber (volume 500 µl). The tissue was held in position with two electron microscopy grids weighed down by

short pieces of platinum wire. The slice was completely immersed in a continuously flowing (~2 ml/min), heated solution (35°C) of the same composition as indicated above. Most recordings of dopaminergic neurons were made from neurons located in the substantia nigra pars compacta. Identification of dopaminergic and CA1 pyramidal cells was performed as described previously (Scuvée-Moreau et al., 1997; Seutin et al., 1997).

Intracellular recordings were made using glass microelectrodes filled with 2 M KCl (resistance 70 to 150 MΩ). All recordings were made in the bridge balance mode, using an NPI SEC1L amplifier (NPI Electronic GmbH, Tamm, Germany). The accuracy of the bridge was checked throughout the experiment by examining the voltage deflection induced by a small (–50 pA) current injection. The potential of the extracellular medium was measured at the end of each experiment, and its absolute value was within 5 mV of that set to 0 at the start. Membrane potentials and injected currents were recorded on a Gould TA240 chart recorder (Gould Instrument Systems, Valley View, OH) and on a Fluke Combiscope oscilloscope (Fluke Corp., Everett, WA). The Flukeview software was used for off-line analysis in most cases. Some experimental data were recorded with pClamp (Axon Instruments, Inc., Foster City, CA).

Drug effects on the prominent apamin-sensitive AHP in dopaminergic neurons were quantified as the percentage of reduction of the surface area of the AHP (in millivolts per second), which was blocked by a maximally active concentration of apamin (300 nM; Seutin et al., 1997). Averages of four sweeps were considered in all cases. In these experiments, the spontaneous firing of the neurons was usually reduced by constant current injection (–20 to –100 pA) to increase the amplitude of the AHP. Because the amplitude of the AHP is very sensitive to the firing rate, care was taken to compare all AHPs of one cell at the same firing rate; this usually necessitated only very small adjustments of the injected currents (i.e., less than 20 pA). Excitability of dopaminergic neurons and CA1 pyramidal cells was assessed by applying long (≥1 s) depolarizing pulses of increasing intensities, at about 4- to 5-s intervals, and counting the number of action potentials elicited by the different pulses; averages of four pulses of similar intensity were considered in the analysis of the results. In these experiments, the baseline membrane potential was set at –60 to –65 mV by negative current injection.

The antagonism at GABA<sub>A</sub> receptors was quantified in dopaminergic cells as the ability to antagonize the reduction in input resistance induced by 3 µM muscimol. Input resistance was measured by the amplitude of the steady-state voltage deflection elicited by passing a small hyperpolarizing current (–20 to –60 pA). These experiments were performed in the presence of tetrodotoxin (0.5 µM) to minimize indirect effects. Cesium (3 mM) or ZD7288 [4-(*N*-ethyl-*N*-phenylamino)-1,2-dimethyl-6-(methylamino)-pyrimidin-2-yl] chloride (30 µM) were used in these experiments to block the activation of the I<sub>h</sub> current (Harris and Constanti, 1995; Mercuri et al., 1995). All drugs were applied by superfusion. Complete exchange of the bath solution occurred within 2 to 3 min.

Curve fitting was carried out using Kaleidagraph (Synergy Software, Reading, PA) or GraphPad Prism (GraphPad Software, Inc., San Diego, CA) and the standard equation,  $E = E_{\max}/[1 + (IC_{50}/x)^h]$ , where  $x$  is the concentration of the drug and  $h$  is the Hill coefficient. Numerical values are expressed as means ± S.E.M. Statistical analysis was performed using Student's *t* test. The level of significance was set at  $p < 0.05$ .

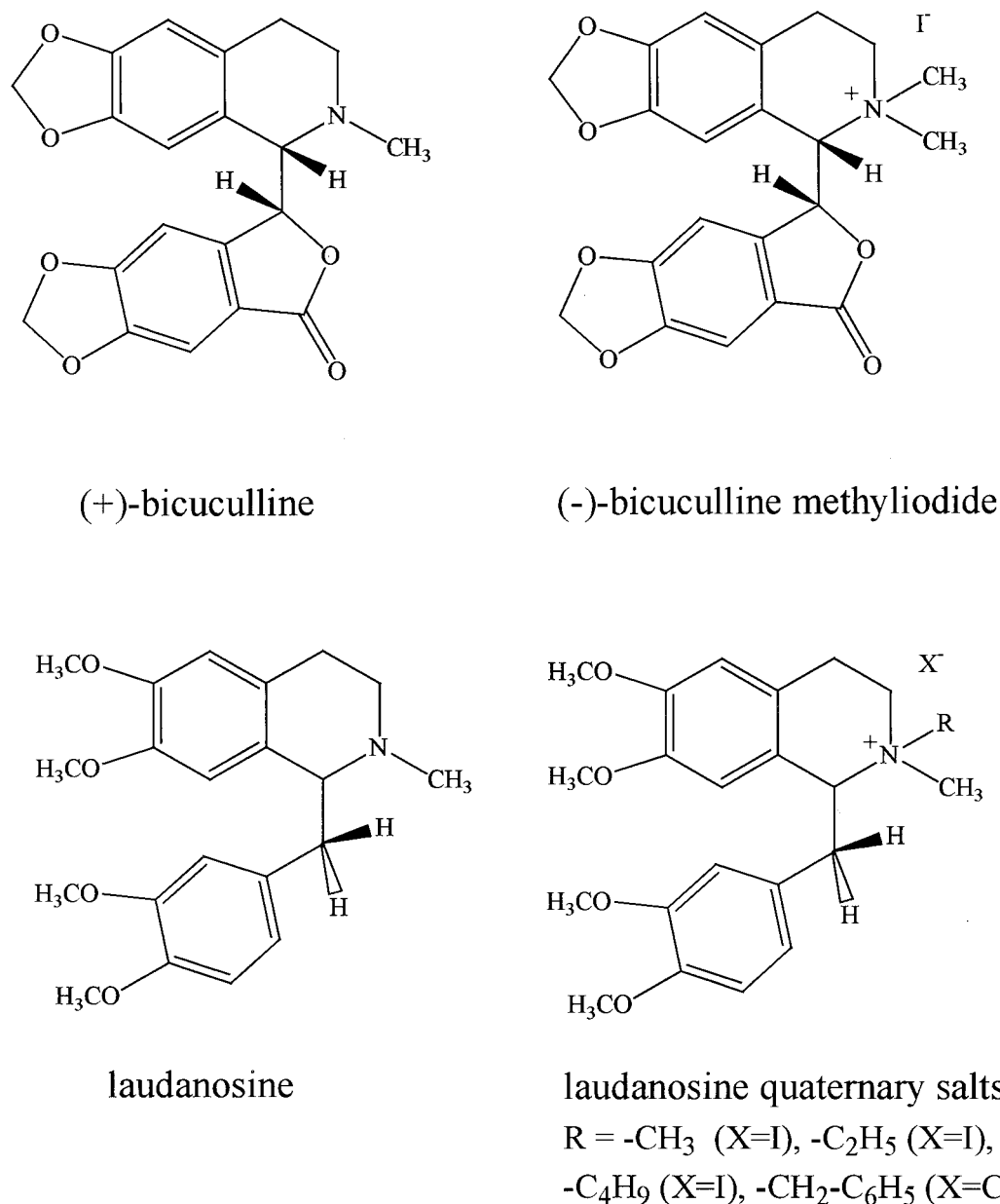
**Binding Studies.** Radioligand binding studies were performed to assess the affinity of laudanosine and its quaternary derivatives for SK channels and the affinity of methyl-laudanosine for voltage-gated potassium channels and muscarinic and nicotinic receptors. Assays were performed by (Cerep/Paris, France) on rat cerebral cortex membranes using general procedures described by Hugues et al. (1982) for SK channels, by Sorensen and Blaustein (1989) for voltage-gated potassium channels, by Richards (1990) for nonselective muscarinic receptors, by Pabreza et al. (1991) for neuronal α-bungarotoxin-insensitive

nicotinic receptors, and by Sharples et al. (2000) for neuronal  $\alpha$ -bungarotoxin-sensitive nicotinic receptors.

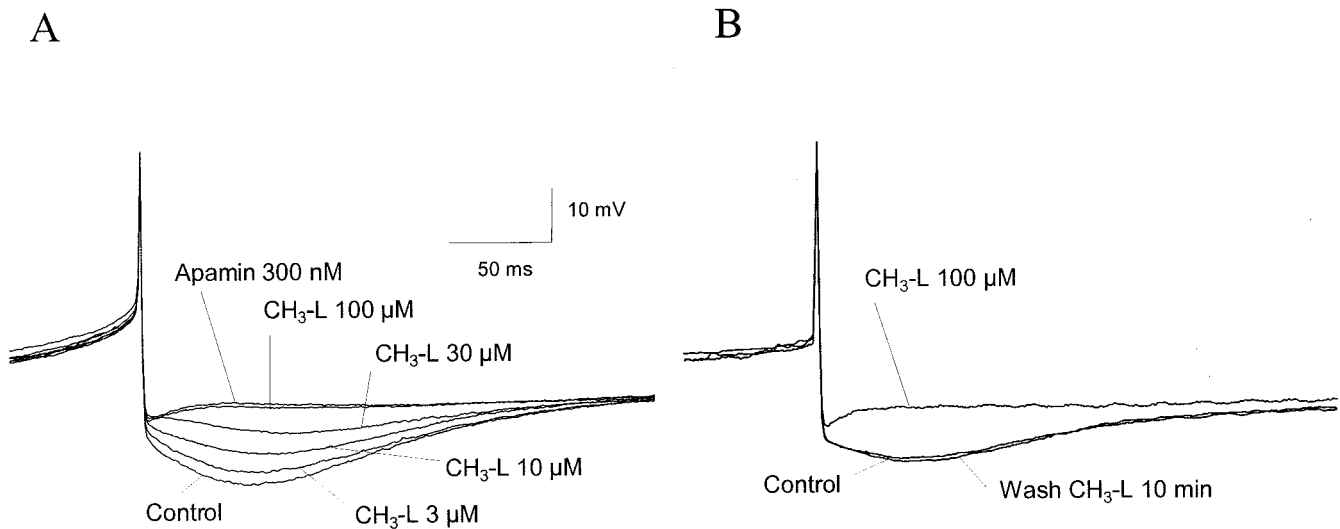
Ligands used and incubation conditions are as follows: 0.004 nM  $^{125}$ I-apamin, 30 min/0°C (SK channels); 0.01 nM  $^{125}$ I- $\alpha$ -dendrotoxin, 30 min/22°C (voltage-gated potassium channels), 0.05 nM [ $^3$ H]3-quinuclidinylbenzilate, 120 min/22°C (muscarinic receptors); 1.5 nM [ $^3$ H]cytisine, 75 min/4°C ( $\alpha$ -bungarotoxin-insensitive nicotinic receptors); and 1 nM  $^{125}$ I- $\alpha$ -bungarotoxin, 150 min/37°C ( $\alpha$ -bungarotoxin-insensitive nicotinic receptors). Nonspecific binding was assessed using 0.1  $\mu$ M apamin, 50 nM  $\alpha$ -dendrotoxin, 1  $\mu$ M atropine, 10  $\mu$ M nicotine, and 10  $\mu$ M  $\alpha$ -bungarotoxin, respectively. Following incubation, membranes were rapidly filtered under vacuum through glass fiber filters (GF/B, PerkinElmer Life Sciences, Boston, MA; GF/C, Whatman International, Maidstone, UK; or Filtermat A or B, PerkinElmer Wallac, Gaithersburg, MD). Filters were then washed several times with an ice-cold buffer using a cell harvester (Packard; Brandel Inc., Gaithersburg, MD; or Tomtec, Orange, CT). Bound radioactivity was measured with a scintillation counter (Topcount, Packard; LS series, Beckman Coulter, Inc., Fullerton, CA; or Beta-plate, Wallac) using a liquid scintillation cocktail (Microscint 0 or

Formula 989, Packard) or a solid scintillant (Meltilex B/HS, Wallac). Laudanosine derivatives were tested in triplicate on SK channels; methyl-laudanosine was tested in triplicate on voltage-gated potassium channels and in duplicate on muscarinic and nicotinic receptors. In each experiment, the respective reference compound was tested at a minimum of eight concentrations in duplicate to obtain a competition curve to validate the experiment. The specific radioligand binding to the receptors is defined as the difference between total binding and nonspecific binding determined in the presence of an excess of unlabeled ligand.  $IC_{50}$  values and Hill coefficients were determined fitting the data to the Hill equation using nonlinear regression analysis. The inhibition constants ( $K_i$ ) were calculated from the Cheng-Prusoff equation ( $K_i = IC_{50}/(1 + L/K_D)$ , where  $L$  is the concentration of radioligand in the assay and  $K_D$  is the affinity of the radioligand for the receptor).

**Compounds.** Methyl-laudanosine iodide, ethyl-laudanosine iodide, butyl-laudanosine iodide, and benzyl-laudanosine chloride (Fig. 1) were synthesized in our laboratory according to conventional methods. Briefly, a mixture of laudanosine with an excess of alkyl or aralkyl halide in acetonitrile was refluxed overnight. Excess of reagent and



**Fig. 1.** Chemical structure of bicuculline, laudanosine, and their quaternary salts, bicuculline methyl iodide, and laudanosine methyl iodide ( $R = -CH_3$ ), ethyl iodide ( $R = -C_2H_5$ ), butyl iodide ( $R = -C_4H_9$ ), and benzyl chloride ( $R = -CH_2-C_6H_5$ ).



**Fig. 2.** Effect of increasing concentrations of methyl-laundanosine (CH<sub>3</sub>-L) on the AHP of dopaminergic neurons. A maximal block similar to the one obtained with 300 nM apamin is obtained with 100 μM CH<sub>3</sub>-L (A). The effect of CH<sub>3</sub>-L is fully reversible after 10 min in wash (B). Each trace is the mean of four sweeps. Action potentials are truncated.

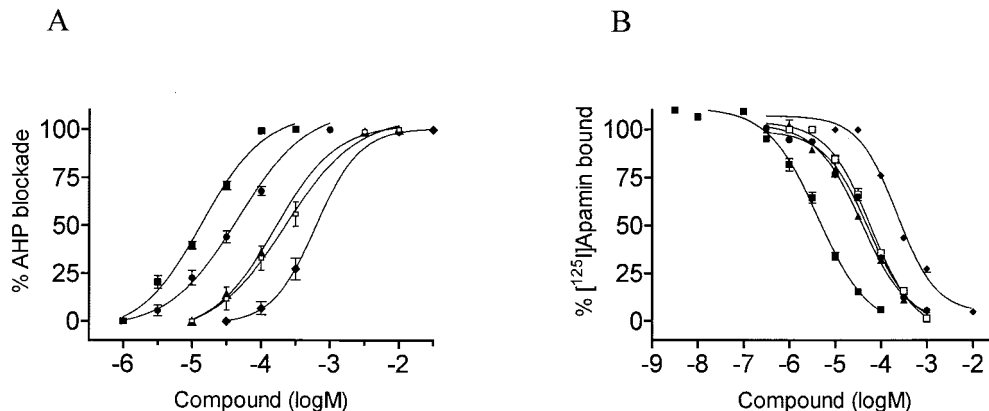
solvent was removed under reduced pressure. For each compound, the crude quaternary salt was isolated in an acetone/diethyl ether mixture. Finally, the products were recrystallized from ethanol, methyl ethyl ketone, diethyl ether/acetone, or dichloromethane/diethyl ether for methyl, ethyl, butyl, and benzyl derivatives, respectively. Purity was checked with classical methods, such as elemental analysis, mass spectrometry, and determination of the melting point.

Other compounds used and their sources were as follows: apamin, cesium chloride, atropine sulfate, (–)nicotine bitartrate, α-dendrotoxin, α-bungarotoxin (obtained from Sigma-Aldrich, St. Louis, MO), laudanosine (purchased from Aldrich Chemical Co., Milwaukee, WI), muscimol, tetrodotoxin (obtained from Tocris Cookson Inc., Ballwin, MO), <sup>125</sup>I-apamin, [<sup>3</sup>H]quinuclidinyl benzilate, [<sup>3</sup>H]cytisine, <sup>125</sup>I-α-bungarotoxin (purchased from PerkinElmer Life Sciences, Boston, MA), <sup>125</sup>I-α-dendrotoxin (purchased from Amersham Biosciences Inc., Piscataway, NJ), ZD7288 (gift from AstraZeneca Pharmaceuticals LP, Wilmington, DE), and SR95531 [2-[carboxy-3'-propyl]-3-amino-6-paramethoxy-phenyl-pyridazinium bromide] (gift from Sanofi-Synthelabo, Paris, France). Laudanosine was dissolved in ethanol or dimethyl sulfoxide; all other drugs were dissolved in water.

## Results

**Midbrain Dopaminergic Neurons.** All recorded neurons ( $n = 32$ ) displayed characteristic features of identified dopamine neurons (Grace and Onn, 1989), including broad-action potentials ( $\geq 1$  ms at 50% maximum height) with a threshold close to  $-40$  mV, a prominent  $I_h$  current, and a slow AHP, which reached its peak  $\pm 50$  ms after the action potential; their mean input resistance was  $183 \pm 11$  MΩ.

Methyl-laundanosine induced a marked blockade of the slow AHP; this effect was concentration-dependent, reaching a maximum at 100 μM. The  $IC_{50}$  and the Hill coefficient were  $15 \pm 1$  μM and  $1.1 \pm 0.03$  ( $n = 7$ ), respectively. The maximal effect, complete block of the AHP, was similar to that seen with 300 nM apamin (Fig. 2A). The effect of methyl-laundanosine was fully and quickly reversible (about 10 min for complete recovery) (Fig. 2B), contrary to what is observed with apamin (Seutin et al., 1993). Ethyl-laundanosine also blocked the apamin-sensitive AHP in a concentration-depen-



**Fig. 3.** A, concentration-response curves for the blockade of the slow AHP in midbrain dopaminergic neurons by laudanosine derivatives. Each data point is presented as the mean  $\pm$  S.E.M. of three experiments except for methyl- and ethyl-laundanosine ( $n = 7$  and  $n = 6$ , respectively). Values for concentrations higher than 300 μM were extrapolated. B, displacement of <sup>125</sup>I-apamin binding at rat neocortical SK channels by laudanosine derivatives. Each data point is presented as the mean  $\pm$  S.E.M. of three experiments. Values for concentrations higher than 1 mM were extrapolated. ■, Methyl-laundanosine; ●, ethyl-laundanosine; □, benzyl-laundanosine; ◆, butyl-laundanosine; ▲, laudanosine.



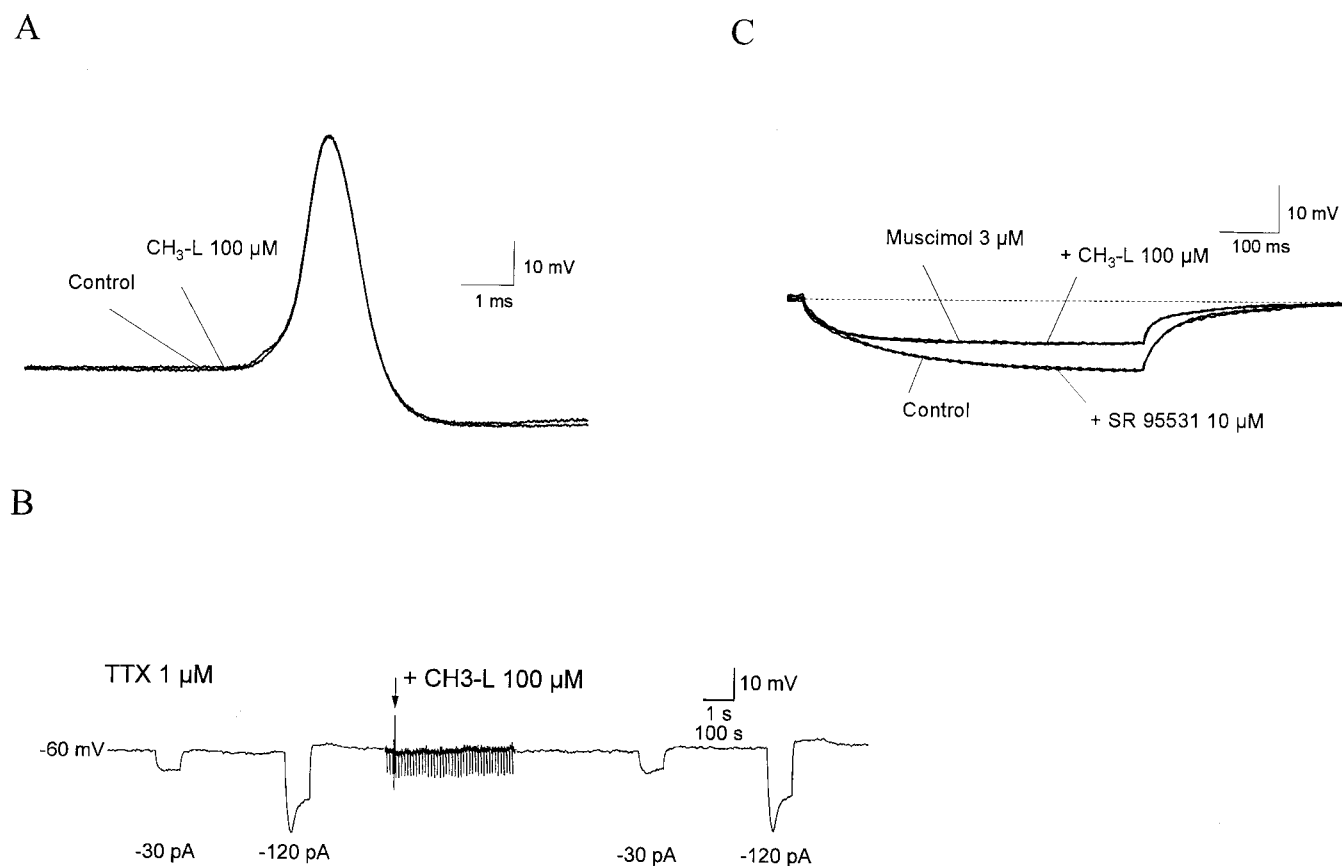
dent manner, but it was less potent than methyl-laudanosine. Its  $IC_{50}$  and Hill coefficient were  $47 \pm 6 \mu\text{M}$  and  $1.0 \pm 0.02$  ( $n = 6$ ), respectively. The effect of ethyl-laudanosine was also fully and quickly reversible. Laudanosine, benzyl-laudanosine, and butyl-laudanosine had a weak inhibitory effect on the slow AHP, with respective  $IC_{50}$  values of  $152 \pm 8 \mu\text{M}$ ,  $249 \pm 39 \mu\text{M}$ , and  $>300 \mu\text{M}$  ( $n = 3$  for each compound). Results are summarized in Fig. 3A.

Because methyl-laudanosine was the most potent blocker of the AHP among the various laudanosine derivatives tested, its influence on different electrophysiological parameters was further investigated. Methyl-laudanosine ( $3\text{--}100 \mu\text{M}$ ) had no effect on the amplitude ( $50 \pm 2.5 \text{ mV}$  from threshold) and the duration of the action potential ( $1.3 \pm 0.2 \text{ ms}$  at mid-height in both control and treatment conditions) ( $n = 4$ ). Its effect on membrane potential, input resistance, and  $I_h$  current was examined in four cells (three in the presence of  $1 \mu\text{M}$  tetrodotoxin). For this purpose, neurons were hyperpolarized to  $-60 \text{ mV}$  by continuous current injection ( $-200$  to  $-300 \text{ pA}$ ), their resistance was estimated by measuring the voltage deflection induced by a small injection of current ( $-20$  to  $-40 \text{ pA}$ ), and a robust  $I_h$  current was elicited by current injections of  $-80$  to  $-120 \text{ pA}$ . Methyl-laudanosine had no effect on membrane potential, input resistance ( $231 \pm 22 \text{ M}\Omega$  in both control and treatment condi-

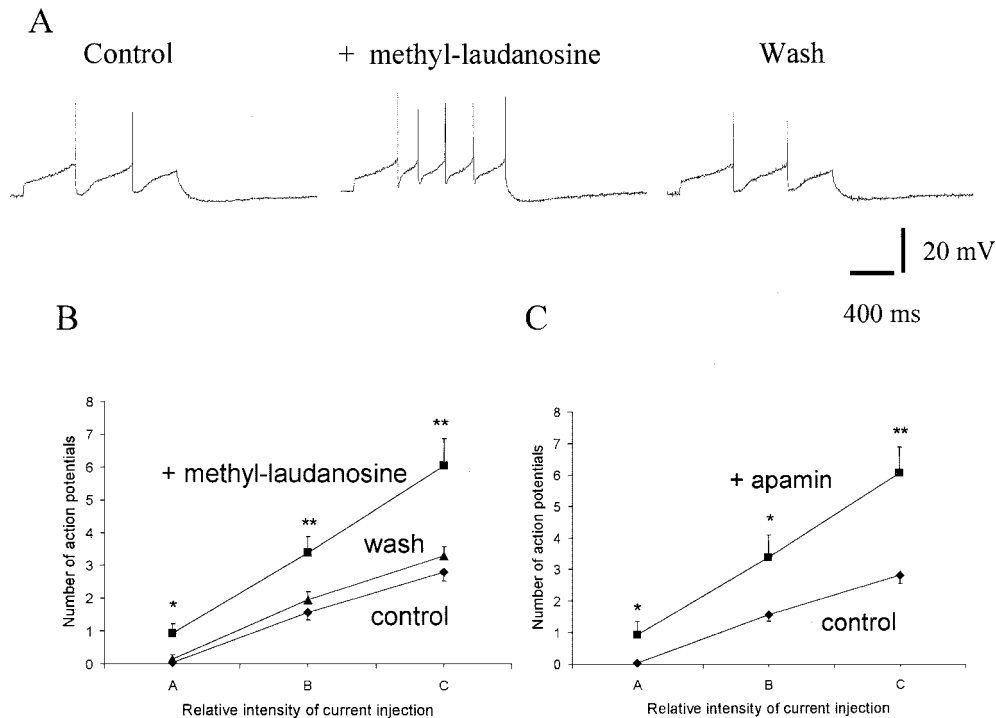
tions), or on the voltage deflection induced by activation of  $I_h$  ( $10 \pm 0.6 \text{ mV}$ ) (Fig. 4, A and B). A putative effect of methyl-laudanosine on  $\text{GABA}_A$  receptors was also investigated in four cells; methyl-laudanosine ( $100 \mu\text{M}$ ) did not modify the decrease in input resistance induced by muscimol ( $3 \mu\text{M}$ ), whereas the  $\text{GABA}_A$  antagonist SR95531 ( $10 \mu\text{M}$ ) completely reversed the effect of muscimol (Fig. 4C).

The consequences of AHP blockade by methyl-laudanosine on cell excitability were investigated by applying long ( $1\text{--}2 \text{ s}$ ) depolarizing current pulses of increasing intensities from a resting membrane potential set at about  $-60 \text{ mV}$ . The effect of methyl-laudanosine was compared with that of apamin. Under control conditions, tonic firing (1 to 4 spikes) was induced by depolarizing pulses of increasing intensities ( $50$  to  $200 \text{ pA}$ ). Both  $100 \mu\text{M}$  methyl-laudanosine ( $n = 8$ ) and  $300 \text{ nM}$  apamin ( $n = 6$ ) induced a significant increase in the number of spikes evoked in the recorded cells; the effect of methyl-laudanosine was reversible after 10 to 20 min in wash (Fig. 5A). The effect of both compounds was independent of the amplitude of the injected current (Fig. 5, B and C).

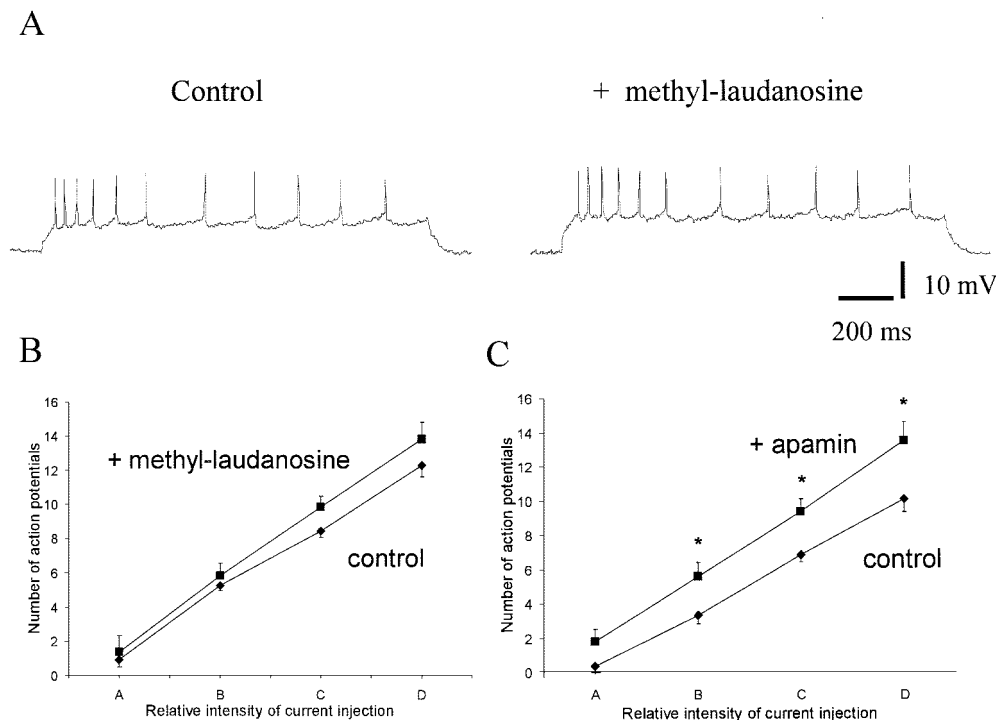
**Hippocampus CA1 Pyramidal Cells.** In view of the different regional distribution of SK subunits, it was interesting to investigate the influence of methyl-laudanosine on the excitability of CA1 pyramidal cells, as these cells have a high level of expression for SK1 and SK2 subunits, contrary



**Fig. 4.** Effect of  $100 \mu\text{M}$  methyl-laudanosine ( $\text{CH}_3\text{-L}$ ) on cell parameters and  $\text{GABA}_A$  receptors in dopaminergic neurons recorded intracellularly in rat brain slices. **A**,  $\text{CH}_3\text{-L}$  has no effect on spike amplitude or duration. **B**,  $\text{CH}_3\text{-L}$  has no effect on membrane potential, on input resistance measured by the amplitude of the voltage deflection elicited by a hyperpolarizing pulse of  $-30 \text{ pA}$ , or on the  $I_h$  current activated by a hyperpolarizing pulse of  $-120 \text{ pA}$ . The experiment was performed in the presence of  $0.5 \mu\text{M}$  tetrodotoxin (TTX); chart speed was reduced during 7 min at the beginning of drug application. **C**,  $\text{CH}_3\text{-L}$  does not modify the decrease in input resistance induced by muscimol, whereas this decrease is fully antagonized by the  $\text{GABA}_A$  antagonist SR95531. Traces are means of voltage deflections induced by a current injection of  $-60 \text{ pA}$  from a potential of  $-60 \text{ mV}$  in control and different treatment conditions. ZD7288 ( $30 \mu\text{M}$ ) and tetrodotoxin ( $0.5 \mu\text{M}$ ) were present throughout the experiment.



**Fig. 5.** Effect of methyl-laundanosine and apamin on the excitability of midbrain dopaminergic neurons. A, action potentials (truncated in the figure) were elicited by 200 pA depolarizing current injections before, during, and after application of 100  $\mu$ M methyl-laundanosine; the membrane potential was set at  $-60$  mV by a continuous injection of  $-100$  pA. B and C, the number of action potentials elicited by 1- to 1.5-s current injections are plotted against the level of intensity of the injected current. Methyl-laundanosine (100  $\mu$ M) (B) and apamin (300 nM) (C) induced a significant increase in the number of action potentials evoked (\*,  $p < 0.05$ ; \*\*,  $p < 0.005$ ). The effect of methyl-laundanosine was reversible (see A). Values are means of eight (methyl-laundanosine) and six (apamin) experiments. Absolute values of current injection were different among experiments; the intensities were chosen to produce increasing numbers of action potentials from 0 to 4 per injection in control conditions.



**Fig. 6.** Effect of methyl-laundanosine and apamin on the excitability of hippocampus CA1 pyramidal cells. A, trains of action potentials (truncated in the figure) characterized by early- and late-frequency adaptation were elicited by 150 pA depolarizing current injections; resting membrane potential was  $-65$  mV. Methyl-laundanosine (100  $\mu$ M) did not modify cell excitability. B and C, the number of action potentials elicited by  $\sim 1$ -s current injections are plotted against the level of intensity of the injected current. Methyl-laundanosine (100  $\mu$ M) (B) failed to modify the number of action potentials evoked, whereas apamin (300 nM) (C) induced a significant increase in this number (\*,  $p < 0.05$ ). Values are means of eight experiments. Absolute values of current injection were different among experiments; the intensities were chosen to produce increasing numbers of action potentials (from 0 to 12) in control conditions.

TABLE 1

Binding affinities of laudanosine, its quaternary derivatives, and the reference compound, apamin, at SK channels

The  $K_i$  values were calculated by the method of Cheng and Prusoff.

Compound	SK Channel		
	IC <sub>50</sub>	K <sub>i</sub>	<i>h</i>
	<i>nM</i>		
Test compound			
Laudanosine	40,200	30,400	0.9
Methyl-laudanosine	3,850	2,450	0.8
Ethyl-laudanosine	47,800	30,400	0.9
Butyl-laudanosine	~294,000*	N.C.	N.C.
Benzyl-laudanosine	56,400	35,900	1.0
Reference compound			
Apamin	0.015	0.0095	1.0

IC<sub>50</sub>, concentration causing a half-maximal inhibition of control-specific binding; K<sub>i</sub>, inhibition constant; *h*, Hill coefficient; N.C., not calculable.

\* This value is approximative; a precise IC<sub>50</sub> value could not be obtained.

to midbrain dopaminergic neurons, which express mostly the SK3 subunit (Stocker and Pedarzani, 2000). Experiments were performed on eight cells; their mean input resistance was  $55 \pm 7.5$  M $\Omega$ . Under control conditions, long (~1 s) depolarizing pulses elicited trains of action potentials characterized by early- and late-spike frequency adaptation (Fig. 6A, left panel). Methyl-laudanosine (100  $\mu$ M) failed to significantly modify spiking frequency and pattern (Fig. 6, A and B), whereas apamin (300 nM) induced a significant increase of the number of action potentials evoked without modifying the late-spike frequency adaptation (not shown). The effect of apamin was independent of the amplitude of the injected current (Fig. 6C).

**Binding Studies.** The IC<sub>50</sub> and K<sub>i</sub> values determined for laudanosine, its quaternary derivatives, and the reference compound apamin at neocortical SK channels are presented in Table 1 and Fig. 3B. The order of affinity was methyl-laudanosine > laudanosine  $\geq$  ethyl-laudanosine  $\geq$  benzyl-laudanosine > butyl-laudanosine. The IC<sub>50</sub> and K<sub>i</sub> values determined for methyl-laudanosine and the reference compounds at voltage-gated potassium channels and muscarinic and nicotinic receptors are shown in Table 2. Depending on the parameter being considered, the affinity of methyl-laudanosine for SK channels was 8 to 30 times superior to its affinity for muscarinic receptors. The affinity for nicotinic receptors was weak or negligible depending upon the subtype examined ( $\alpha$ -bungarotoxin-insensitive or -sensitive). No affinity for voltage-gated potassium channels could be detected.

TABLE 2

Binding affinities of methyl-laudanosine and reference compounds at voltage-gated potassium channels, and muscarinic and nicotinic receptors

The K<sub>i</sub> values were calculated by the method of Cheng and Prusoff.

Receptor	Methyl-Laudanosine				Reference Compounds		
	IC <sub>50</sub>	K <sub>i</sub>	<i>h</i>		IC <sub>50</sub>	K <sub>i</sub>	<i>h</i>
	$\mu$ M				<i>nM</i>		
Voltage-gated potassium channel	>1,000	N.C.	N.C.	$\alpha$ -Dendrotoxin	0.53	0.42	1.2
Muscarinic (nonselective)	114	19.1	0.8	Atropine	0.37	0.062	0.9
Nicotonic (neuronal) ( $\alpha$ -bungarotoxin-insensitive)	>1,000	N.C.	N.C.	Nicotine	16	8.9	1.0
Nicotinic (neuronal) ( $\alpha$ -bungarotoxin-sensitive)	367	84.7	1.0	$\alpha$ -Bungarotoxin	3.8	0.89	0.6

IC<sub>50</sub>, concentration causing a half-maximal inhibition of control-specific binding; K<sub>i</sub>, inhibition constant; *h*, Hill coefficient; N.C. value not calculable because of insufficient inhibition at the highest test concentration (1 mM).

## Discussion

This study was initiated to identify a specific and reversible blocker of SK channels. Drawbacks of blockers such as apamin, bicuculline, (+)-tubocurarine, and gallamine have been stressed earlier (see the Introduction).

Our results show that quaternary laudanosine derivatives differentially block the slow AHP in rat dopaminergic neurons, with the *N*-methyl derivative being the most potent blocker. This property, which was previously described for quaternary salts of bicuculline, namely, bicuculline methyl iodide and bicuculline methochloride (Seutin et al., 1997), was suspected given the similarity of structure between these compounds. Methyl-laudanosine is slightly more potent as a blocker of the AHP than bicuculline salts, whose IC<sub>50</sub> was found to be 26  $\mu$ M in the same conditions (Seutin et al., 1997). The difference of potency (~10 times) between the methyl derivative of laudanosine and the base is similar to that reported previously between the quaternary salts of bicuculline and the base (Seutin et al., 1997). Increasing the length of the *N*-substituent does not increase the activity on SK channels.

Experiments on cell excitability show that apamin and methyl-laudanosine significantly increase the number of spikes evoked in midbrain dopaminergic neurons by positive current injection. These results are consistent with other studies indicating a role for SK channels in the control of the firing frequency of these cells (Shepard and Bunney, 1991; Wolfart et al., 2001). Interestingly, an increase in the firing frequency of hippocampus CA1 pyramidal cells was observed with apamin but not with methyl-laudanosine. Our results with apamin are in agreement with previous studies indicating a contribution of apamin-sensitive SK channels to the firing properties of hippocampal pyramidal neurons (Stocker et al., 1999). The fact that methyl-laudanosine does not modify cell excitability at a concentration inducing a maximal block of the AHP in dopaminergic neurons suggests the possibility that this compound discriminates between SK subtypes. In fact, recent studies show a high level of expression of SK3 subunits in midbrain dopaminergic neurons (Tacconi et al., 2001; Wolfart et al., 2001) and a high level of expression of SK1 and SK2 subunits in CA1 to CA3 pyramidal neurons (Stocker et al., 1999). Electrophysiological results thus point to a preferential effect of methyl-laudanosine on the SK3 subtype of SK channels. However, further studies in slices and in cell lines expressing the various subunits are needed to address this point thoroughly.

Binding data obtained from rat cerebral cortex membranes are in good agreement with the electrophysiological study on

dopaminergic cells and confirm the good affinity of methyl-laudanosine for SK channels. In fact,  $IC_{50}$  values obtained in the two kinds of studies are quite similar ( $\sim 15 \mu M$  in electrophysiological studies and  $\sim 4 \mu M$  in binding experiments). Furthermore, the difference of potency ( $\sim 10$  times) between laudanosine and its methyl derivative is also similar in the two studies. With the exception of ethyl-laudanosine, the order of affinity for SK channels among laudanosine derivatives (methyl-laudanosine > laudanosine  $\geq$  ethyl-laudanosine  $\geq$  benzyl-laudanosine > butyl-laudanosine) is comparable with their order of potency as AHP blockers (methyl-laudanosine > ethyl-laudanosine > laudanosine > benzyl-laudanosine > butyl-laudanosine). As pointed out earlier, this may be due to the differences in SK subtypes that exist between brain regions.

Additional data from our study show that methyl-laudanosine has no affinity for  $GABA_A$  and nicotinic receptors. This is an interesting finding because pharmacological studies suggest that the tridimensional structure of the binding site in SK,  $GABA_A$ , and nicotinic channels is similar (Seutin and Johnson, 1999). Furthermore, methyl-laudanosine is less potent at muscarinic receptors than at SK channels. Indeed, more recent iontophoretic experiments performed in vivo in our laboratory suggest that local effects of methyl-laudanosine are not due to muscarinic effects (Seutin V., Massotte L., Liégeois J.-F., and Scuvée-Moreau J., unpublished results). Finally, both our electrophysiological and binding studies suggest that methyl-laudanosine does not interact with fast  $Na^+$  channels, voltage-dependent  $K^+$  channels, or  $I_h$  channels. It should be noted, however, that we cannot exclude an action of the compound on another receptor or channel.

Methyl-laudanosine has a relatively low potency when compared with apamin or UCL1684. This is probably due to a high off-rate of the compound from the channel that makes it a rapidly reversible blocker. This property may be interesting for pharmacological experiments in which a relatively quick reversal of the effect is needed. Again, our recent in vivo iontophoretic experiments show that this is the case.

Our study demonstrates the pharmacological differences between bicuculline quaternary salts and methyl-laudanosine. Additional modifications of this alkaloid will be performed to further study the structural elements required for a putative selective action on SK-channel subtypes.

In conclusion, the lack of influence of methyl-laudanosine on various neuronal parameters combined with its lack of antagonism at  $GABA_A$  receptors and its lack of affinity for nicotinic receptors suggest that this agent may become an interesting tool to examine the functional role of SK channels in various experimental conditions.

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