British Journal of Pharmacology (2004) 143, 753–764
www.nature.com/bjp

Electrophysiological characterization of the SK channel blockers methyl-laudanosine and methyl-noscapine in cell lines and rat brain slices

[1,4]Jacqueline Scuvée-Moreau, [1,4]Andre Boland, [2]Amaury Graulich, [1]Lionel Van Overmeire, [3]Dieter D'hoedt, [1]Fabienne Graulich-Lorge, [1]Elizabeth Thomas, [1]Aude Abras, [3]Martin Stocker, [2]Jean-Francois Liégeois & *,[1]Vincent Seutin

[1]Research Center for Cellular and Molecular Neurobiology and Laboratory of Pharmacology, University of Liège, B-4000 Liège, Belgium; [2]Laboratory of Medicinal Chemistry and Natural and Synthetic Drugs Research Center, University of Liège, B-4000 Liège, Belgium and [3]Wellcome Laboratory for Molecular Pharmacology, Department of Pharmacology, University College of London, London WC1E 6BT

1 We have recently shown that the alkaloid methyl-laudanosine blocks SK channel-mediated afterhyperpolarizations (AHPs) in midbrain dopaminergic neurones. However, the relative potency of the compound on the SK channel subtypes and its ability to block AHPs of other neurones were unknown.

2 Using whole-cell patch-clamp experiments in transfected cell lines, we found that the compound blocks SK1, SK2 and SK3 currents with equal potency: its mean IC_{50}s were 1.2, 0.8 and 1.8 μM, respectively. IK currents were unaffected. In rat brain slices, methyl-laudanosine blocked apamin-sensitive AHPs in serotonergic neurones of the dorsal raphe and noradrenergic neurones of the locus coeruleus with IC_{50}s of 21 and 19 μM, as compared to 15 μM in dopaminergic neurones. However, at 100 μM, methyl-laudanosine elicited a constant hyperpolarization of serotonergic neurones of about 9 mV, which was inconsistently (i.e. not in a reproducible manner) antagonized by atropine and hence partly due to the activation of muscarinic receptors.

3 While exploring the pharmacology of related compounds, we found that methyl-noscapine also blocked SK channels. In cell lines, methyl-noscapine blocked SK1, SK2 and SK3 currents with mean IC_{50}s of 5.9, 5.6 and 3.9 μM, respectively. It also did not block IK currents. Methyl-noscapine was slightly less potent than methyl-laudanosine in blocking AHPs in brain slices, its IC_{50}s being 42, 37 and 29 μM in dopaminergic, serotonergic and noradrenergic neurones, respectively. Interestingly, no significant non-SK effects were observed with methyl-noscapine in slices. At a concentration of 300 μM, methyl-noscapine elicited the same changes in excitability in the three neuronal types than did a supramaximal concentration of apamin (300 nM).

4 Methyl-laudanosine and methyl-noscapine produced a rapidly reversible blockade of SK channels as compared with apamin. The difference between the IC_{50}s of apamin (0.45 nM) and methyl-laudanosine (1.8 μM) in SK3 cells was essentially due to a major difference in their k_{-1} (0.028 s^{-1} for apamin and $\geqslant$20 s^{-1} for methyl-laudanosine).

5 These experiments demonstrate that both methyl-laudanosine and methyl-noscapine are medium potency, quickly dissociating, SK channel blockers with a similar potency on the three SK subtypes. Methyl-noscapine may be superior in terms of specificity for the SK channels.
British Journal of Pharmacology (2004) **143,** 753–764. doi:10.1038/sj.bjp.0705979

Keywords: SK channels; IK channels; electrophysiology; afterhyperpolarization; monoaminergic neurones; transfected cell lines; patch clamp

Abbreviations: AHP, afterhyperpolarization; AP, action potential; BK, big conductance; CH_3-L, methyl-laudanosine; CH_3-N, methyl-noscapine; DA, dopaminergic; DR, dorsal raphe; 5-HT, 5-hydroxytryptamine; IC_{50}, concentration of drug producing 50% of the maximal blockade of the SK current (in cell lines) or of the mAHP (in slices); IK, intermediate conductance; mAHP, medium-duration afterhyperpolarization; NA, noradrenergic; SK, small conductance; τ_{off}, time constant of recovery from current blockade; τ_{on}, time constant of onset of current blockade; TTX, tetrodotoxin

Introduction

Ca^{2+}-activated K^+ channels, which are present in most tissues, play fundamental roles in many important physiological processes. Based on their unitary conductance, Ca^{2+}-activated K^+ channels have been classically classified as BK (big conductance), IK (intermediate conductance) and SK

*Author for correspondence at: Laboratory of Pharmacology, University of Liège, Institute of Pathology (B23), B-4000 Sart-Tilman/Liège 1, Belgium; E-mail: V.Seutin@ulg.ac.be
[4]These authors contributed equally to this work.
Advance online publication: 25 October 2004

(small conductance) channels (Latorre *et al*., 1989). The new IUPHAR nomenclature recommends to name them $K_{Ca}1.1$, $K_{Ca}3.1$ and $K_{Ca}2.x$, respectively (Gutman *et al*., 2003). The corresponding genes are KCNMA1 for the main subunit of BK channels, KCNN4 for IK subunits and KCNN1-3 (see below) for SK subunits, according to the HUGO nomenclature (Gutman *et al*., 2003).

Only BK and SK channels are highly expressed within the central nervous system (CNS). BK channels are involved in spike repolarization and in the fast afterhyperpolarization (AHP) which follows the action potential (Sah, 1996; Sah & Faber, 2002). On the other hand, SK channels are involved in the medium duration AHP (mAHP) which lasts for a few hundred milliseconds in many neuronal types and dampens their excitability. They are therefore in a good position to regulate the firing frequency and pattern of these neurones (Sah, 1996; Sah & Faber, 2002).

SK- or $K_{Ca}2.x$-channels are activated by low intracellular concentrations of Ca^{2+} (100 nM – 1 μM) and are not voltage-dependent. Three homologous mammalian SK channel subunits (SK1 or $K_{Ca}2.1$, SK2 or $K_{Ca}2.2$, SK3 or $K_{Ca}2.3$) have been cloned (Köhlez *et al*., 1996). Each of these subunits has six transmembrane domains, a pore-forming region and intracellular N- and C-termini (Köhlez *et al*., 1996; Xia *et al*., 1998). Calmodulin constitutively binds to the proximal part of the C-terminus and acts as the Ca^{2+} sensor (Xia *et al*., 1998). In heterologous expression systems, homo- as well as heteromeric SK channels have been characterized (Ishii *et al*., 1997; Benton *et al*., 2003; Monaghan *et al*., 2004).

The localization of the three SK subunits was studied in the rat brain by *in situ* hybridization (Stocker & Pedarzani, 2000) and immunohistochemistry (Sailer *et al*., 2002). Globally, SK1 and SK2 subunits are highly expressed in cortex and hippocampus, whereas SK3 subunits are particularly abundant in subcortical areas, especially in the monoamine cell group regions (substantia nigra, dorsal raphe, locus coeruleus).

Electrophysiological whole-cell patch-clamp studies, combined in some cases with single-cell RT–PCR, are consistent with this conclusion. Indeed, as judged by its sensitivity to apamin, there is strong evidence that a major fraction of the mAHP of hippocampal CA1 neurones is mediated by channels comprising SK2 subunits (Stocker *et al*., 1999). On the other hand, it seems that SK3 subunits play a major role in the mAHP of midbrain dopaminergic neurones (Wolfart *et al*., 2001) and dorsal vagal motoneurones (Pedarzani *et al*., 2000) (see Stocker *et al*., 2004 for further details).

Increasing evidence suggests that SK channel modulation (by blockers or enhancers) may be useful in various types of CNS disorders (reviewed in Liégeois *et al*., 2003). For example, the classical peptidic SK blocker apamin has been shown to improve memory in several paradigms (Stackman *et al*., 2002) and to reverse deficits induced by a partial hippocampal lesion (Ikonen & Riekkinen, 1999). Apamin also has antidepressant effects at low doses (Van der Staay *et al*., 1999). Several (Dror *et al*., 1999; Tsai *et al*., 1999; Miller *et al*., 2001; Ritsner *et al*., 2002) but not all (Antonarakis *et al*., 1999; Bonnet-Brilhault *et al*., 1999) studies, suggest that an association may exist between the SK3 channel gene and schizophrenia. Finally, there is evidence that SK channel enhancers may be useful in neuronal hyperexcitability disorders (Pedarzani *et al*., 2001).

Besides the bee venom toxin apamin, various compounds, both peptidic and nonpeptidic, have been shown to block SK channels. Peptidic compounds include leiurotoxin (Abia *et al*., 1986; Chicchi *et al*., 1988), PO5 (Zerrouk *et al*., 1993), tityus kappa (Legros *et al*., 1996) and tamapin (Pedarzani *et al*., 2002). A clever modification of leiurotoxin yielded a compound called Lei-Dab, which was shown to have a very good subtype selectivity for SK2 channels (Shakkottai *et al*., 2001). Nonpeptidic compounds include d-tubocurarine, bicuculline quaternary salts and bis-quinolinium cyclophanes, such as UCL 1684 (Seutin & Johnson, 1999; Campos Rosa *et al*., 2000; Finlayson *et al*., 2001; Liégeois *et al*., 2003).

Enhancers of these channels include 1-ethyl-2-benzimidazolinone (1-EBIO) (Adeagbo, 1999) and chlorzoxazone (Cao *et al*., 2001). These compounds act by increasing the sensitivity of SK channels to Ca^{2+} (Pedarzani *et al*., 2001).

While studying quaternary analogs of laudanosine, an alkaloid related to bicuculline, we recently demonstrated that the *N*-methyl analog (Figure 1a) blocks SK channels with the highest affinity and a good selectivity *versus* other targets (Scuvée-Moreau *et al*., 2002). Its IC_{50} was found to be 4 μM in binding experiments on brain membranes and it blocked the apamin-sensitive AHP in dopaminergic (DA) neurones of the substantia nigra with an IC_{50} of 15 μM. On the other hand, methyl-laudanosine does not interact with other targets such as nicotinic, $GABA_A$, voltage-gated K^+ channels, but it has some affinity for muscarinic receptors. However, these experiments did not allow to evaluate the potency of the

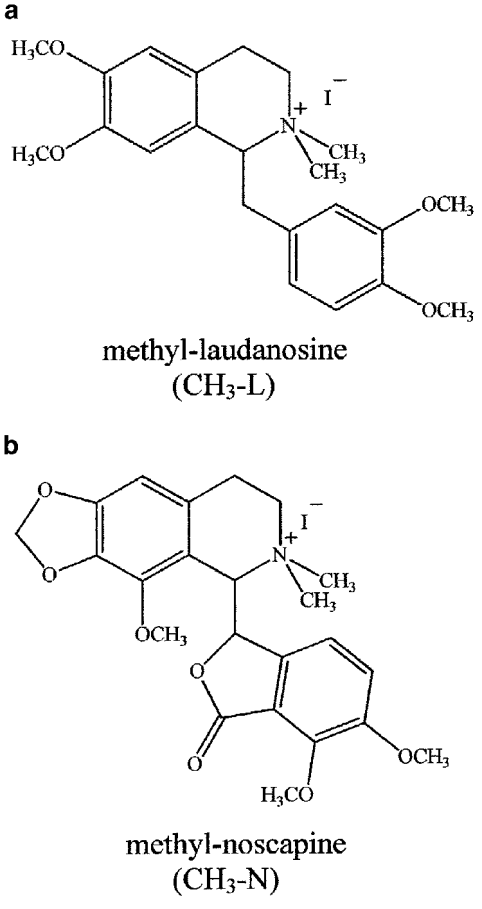

Figure 1 Chemical structures of (a) methyl-laudanosine (CH_3-L) and (b) methyl-noscapine (CH_3-N).

compound on the various SK channel subtypes. Nor did they give an estimate of its on- and off-rates for these channels.

A first aim of this work was to address these questions by using patch-clamp recordings in cell lines, namely Chinese hamster ovary (CHO) cells stably expressing the human SK1 channel or the rat SK2 and SK3 channels, and human embryonic kidney (HEK293) cells expressing IK1. In a next step, we analyzed the effects of this compound on other monoaminergic neurones in slices and found that it has a significant non-SK-mediated effect on serotonergic (5-HT) neurones of the dorsal raphe, but not on noradrenergic (NA) neurones of the locus coeruleus. We therefore synthesized an analog, methyl-noscapine (Figure 1b), which we found to be devoid of this non-SK-related action. We present here its first electrophysiological characterization, both in cell lines and in brain slices.

Methods

Cell culture and SK channel stable transfection

CHO-FlipIn cells were cultured in Ham's F-12 medium supplemented with penicillin/streptomycin, 10% fetal calf serum and 100 μg ml^{-1} Zeocin. Cells were grown in a humidified atmosphere (5% CO_2 and 95% air) at 37°C. The CHO-FlipIn system (Invitrogen, Belgium) was used to generate CHO cell lines stably expressing SK channels. The generation of the hSK1-cell line has been previously described (Pedarzani *et al*., 2002). SK2 and SK3 were ligated into the pcDNA5/FRT vector and transfected in CHO FlipIn cells growing at 60–70% confluency. A measure of 0.9 μg of SK2-pcDNA5/FRT or SK3-pcDNA5/FRT were cotransfected with 10 μg pOG44 into the CHO FlipIn cells using LipofectAMINE™ together with the PLUS™ reagent (Invitrogen, Belgium). Transfected cells were selected in medium containing 100 μg ml^{-1} Hygromycin B. Expression of SK channels was confirmed by patch-clamp recordings. Furthermore, the expression of SK2 and SK3 was confirmed by immunocytochemistry. The cell line stably expressing human IK1 channels was a kind gift of W.J. Joiner and L.K. Kaczmarek (Cao & Houamed, 1999).

The stability of expression of SK channels and the absence of cross-contamination of the cell lines was assessed after several months of culturing by standard immunocytochemical methods, using the subunit specific antibodies anti-NSK2 (1 : 1000) or anti-CSK3 (1 : 500). Generation and characterization of the anti-NSK2 antibody have been described (Cingolani *et al*., 2002). The anti-CSK3 antibody was raised against the carboxy-terminus (aa706–719) of SK3 (Accession no. U69884). The polyclonal rabbit antiserum was generated by a standard protocol by Eurogentec (Belgium). Subunit specificity was ensured by transient transfection of SK channel subunits in cell lines, which were assessed by immunocytochemistry and Western analysis. These experiments showed that the degree of expression of SK3 was stable over time and that no cross-contamination occurred.

Patch-clamp recordings in cell lines

Cells plated on a glass coverslip were positioned in a recording chamber and superfused with a solution at a rate of 2 ml min^{-1}. The normal solution (low K^+ solution) contained (in mM): KCl 4, NaCl 140, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10. The pH was adjusted to 7.4 using NaOH and the osmolarity ranged from 295 to 305 mOsm. The superfusate highly concentrated in K^+ (high K^+ solution) contained (in mM): KCl 144, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10. The pH was adjusted to 7.4 using KOH and the osmolarity ranged from 290 to 305 mOsm.

Patch pipettes (borosilicate glass) were pulled using a Sutter P-87 electrode puller and filled with an internal solution consisting of (mM): KCl 130, EGTA 10, $CaCl_2$ 8.751, $MgCl_2$ 1.08, HEPES 10 yielding a free (unchelated) [Ca^{2+}] of 1 μM. The pH was adjusted to 7.2 using KOH and the osmolarity ranged from 275 to 290 mOsm. In a few experiments, the concentration of $CaCl_2$ and $MgCl_2$ were 6.76 and 1.2 mM to yield a free [Ca^{2+}] of 300 nM, or 4.11 and 1.368 mM to yield a free [Ca^{2+}] of 100 nM. The free [Ca^{2+}] were calculated by EqCal (Biosoft). When filled with the internal solution, the patch electrodes had a tip resistance of 3–4 MΩ.

Currents were recorded using standard whole cell voltage clamp recording techniques at room temperature (21–23°C) using a RK-400 Biologic amplifier. Voltage command protocols and data acquisitions were controlled using Wintida (Heka Electronik, Lambrecht, Germany). For the methyl-laudanosine experiments, we used a holding potential of −100 mV and performed 1 s ramps from −100 to +50 mV at 10 s intervals. The protocol was the same for the methyl-noscapine experiments, except that the holding potential was −80 mV and ramps were from −80 to +70 mV. To obtain the reversal potential of the apamin-sensitive current in SK cell lines, we measured membrane currents at various potentials (from −100 to +40 mV, every 20 mV) in the absence and in the presence of 300 nM of the drug. Straight lines were fitted to the data and the intersection between the lines was determined visually.

In the methyl-laudanosine concentration–response experiments, a supramaximal concentration of apamin (300 nM) was applied at the end of each experiment and SK currents were defined as currents that were sensitive to this concentration of apamin. In the methyl-noscapine study, SK currents were defined as currents that were sensitive to 100 μM methyl-laudanosine, since this concentration of the drug produced a block that was equivalent to the one produced by 300 nM apamin (see Results). The mean percentage of the inward current measured at the holding potential that was resistant either to 300 nM apamin or 100 μM methyl-laudanosine was 43, 40 and 25% for SK1, SK2 and SK3 cells, respectively.

Data were stored on the hard drive of a microcomputer. The final series resistance, calculated according to Armstrong and Gilly (Armstrong & Gilly, 1992) did not exceed 7 MΩ. No attempt was made to compensate it electronically. Junction potentials were negligible using the above-specified recording conditions.

In most cases, drugs were applied through a fine needle positioned close to the cell, *via* a series of gravity-fed reservoirs. The superfusion of the drugs was controlled either by Wintida or manually.

For the kinetic experiments, we used a fast superfusion system consisting of two 1.5 mm diameter square capillaries (Bioscience Tools, San Diego, CA, U.S.A.) forming an angle of ~30° and having their tips close together. The two tips were positioned ~200 μm from the recorded cell. The solution

flowing out of one capillary was the same as the general superfusion solution whereas the test solution (i.e. containing a channel blocker) flew from the other one. The system was optimized by using a slightly supra-atmospheric superfusion pressure. Validation experiments using the high and low K^+ solutions showed that the time constant of solution exchange was 14.9 ± 1.0 ms ($n=8$). In order to evaluate on- and off-rates of channel blockers, we measured the kinetics of variation of the holding current at -80 mV. Wash-ins and wash-outs could be well fitted with monophasic exponentials. Data analysis was performed off-line. The following set of validation experiments were performed in order to confirm that the currents were carried by potassium and were Ca^{2+}-dependent.

Untransfected cells did not express significant apamin-sensitive currents. In transfected SK cell lines, the amplitude of the current was dependent upon the free intracellular Ca^{2+} concentration. Indeed, in the presence of 100 nM free Ca^{2+}, the amplitude of the current was -288 ± 72, -312 ± 180, -527 ± 149 pA for SK1, SK2 and SK3, respectively ($n=3$). In the presence of 300 nM Ca^{2+}, the amplitude was -505 ± 267, -483 ± 226, -463 ± 233 pA for SK1, SK2 and SK3, respectively ($n=3$). In the presence of 1 μM free intracellular Ca^{2+}, currents had an average value of -2418 ± 902, -2215 ± 690 and -1783 ± 997 pA, for SK1, SK2 and SK3, respectively. Finally, the reversal potential of SK currents varied according to the Nernst equation for K^+. Given the ionic concentration used in our study, the predicted reversal potential for a K^+ current was -87.7 mV in the low K^+ solution and $+2.6$ mV in the high K^+ solution. Our measurements yielded values of -89.1 ± 4.8 mV ($n=6$) and $+4.7 \pm 8.4$ mV ($n=6$) (data not shown), which were very close to expected values.

IK currents were defined as those that were blocked by 30 μM clotrimazole, a supramaximal concentration for the blockade of these currents (Jensen *et al*., 1998).

Intracellular recordings in brain slices

The method used has been described previously (Seutin *et al*., 1997; Scuvée-Moreau *et al*., 2002). Briefly, male Wistar rats (150–200 g) were housed and handled in accordance with guidelines of the National Institute of Health (NIH Publications No. 85–23, 1985). They were anesthetized with chloral hydrate (400 mg kg^{-1} i.p.) and decapitated. The brain was rapidly removed and placed in cold ($\sim$4°C) artificial cerebrospinal fluid of the following composition (in mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.4, glucose 11, $NaHCO_3$ 18, saturated with 95% O_2 and 5% CO_2 (pH 7.4). A block of tissue containing the midbrain or the pons was placed in a Vibratome (Lancer) filled with the same solution and cut in horizontal (substantia nigra) or transverse (dorsal raphe and locus coeruleus) slices (thickness: 350 μm). 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 an electron microscopy grid weighed down by short pieces of platinum wire. The slice was completely immersed in a continuously flowing ($\sim$2 ml min^{-1}), heated solution (35°C) of the same composition as indicated above. Structures were visualized by transillumination, using a stereomicroscope. The substantia nigra was identified as a semilucent area rostral and caudal to the medial terminal nucleus of the accessory optic tract (Paxinos & Watson, 1986). The dorsal raphe nucleus was identified as a semilucent region dorsal and medial to the *fasciculus longitudinalis medialis* (Paxinos & Watson, 1986). The locus coeruleus, located laterally to the fourth ventricule, was identified by its clearly defined, bright and translucent appearance when viewed with transmitted light (Paxinos & Watson, 1986).

Intracellular recordings were made using glass microelectrodes filled with KCl 2 M (resistance 70–150 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 experiments 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 zero at the start. Membrane potentials and injected currents were recorded on a Gould TA240 chart recorder (Gould Instrument Systems, Valley View, OH, U.S.A.) and on a Fluke Combiscope oscilloscope (Fluke Corp., Everett, WA, U.S.A.). The Flukeview software was used for off-line analysis.

The characteristics of DA neurones recorded intracellularly have been described previously (Seutin *et al*., 1997; Scuvée-Moreau *et al*., 2002). All putative 5-HT neurones displayed characteristic features of these neurones as described previously (Vandermaelen & Aghajanian, 1983), including broad action potentials with a slight shoulder on their falling phase. The majority of the cells were spontaneously active, with a prominent (amplitude measured from the spike threshold: 10–20 mV) AHP which reached its peak 20–40 ms after the action potential and decayed slowly over a period $>$200 ms. Their mean input resistance was 252 ± 20 MΩ ($n=29$). When neurones were not spontaneously active, a continuous small depolarizing current (10–30 pA) was applied in order to evoke spike discharges. In agreement with previous reports on DR 5-HT neurones (Innis *et al*., 1988), superfusion of 5-HT (30–50 μM) induced a marked hyperpolarization (8.4 ± 1 mV) and a decrease in apparent input resistance in all tested neurones ($n=5$).

All presumed NA neurones ($n=23$) were spontaneously active. Their spikes were followed by a prominent (amplitude measured from the spike threshold: 10–20 mV) AHP which reached its peak $\sim$10 ms after the action potential and decayed gradually over a period of 200 ms. The mean input resistance of these neurones was 133 ± 15 MΩ ($n=23$). Previous experiments in our laboratory have shown that such cells are hyperpolarized by α_2 agonists such as clonidine and are therefore probably noradrenergic.

Drug effects on the apamin-sensitive AHP in monoaminergic neurones were quantified as the percent reduction of the surface area of the AHP (in mVs) which was blocked by a largely supramaximal concentration of apamin (300 nM, Seutin *et al*., 1997). Averages of four sweeps were considered in all cases. 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 required only very small adjustments of the injected currents (i.e. less than 20 pA). In these experiments, increasing concentrations of methyl-laudanosine or methyl-noscapine were superfused before the application of apamin.

Excitability of monoaminergic neurons was assessed by applying long (~1 s) depolarizing pulses of increasing intensities at about 4–5 s intervals and counting the number of action potentials elicited by the different pulses. Averages of four pulses of similar intensities were considered in the analysis of the results. Absolute values of injected currents were different among individual cells; intensities were chosen to produce increasing numbers of action potentials from 0 to 6 per pulse in control conditions. Baseline membrane potential was set at −65 mV by negative current injection. In all experiments, we tested one concentration of methyl-noscapine and of apamin which completely blocked the mAHP (300 μM and 300 nM, respectively).

The antagonism at $GABA_A$ receptors was quantified 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). Some experiments were performed in the presence of tetrodotoxin (TTX) (0.5 μM) in order to minimize indirect effects.

Data analysis and statistics

The effect of the channel blockers was quantified according to the Hill equation $E = E_{max}/[1 + (IC_{50}/x)^h]$, where x is the concentration of the drug and h the Hill coefficient.

For the kinetic experiments, data were analyzed according to the following equations (Chen *et al*., 2003):

$$\tau_{on} = 1/(k_{+1} \times [\text{blocker}] + k_{-1}) \quad (1)$$

$$\tau_{off} = 1/k_{-1} \quad (2)$$

with $K_d = k_{-1}/k_{+1}$, and assuming that the IC_{50} of an ion channel blocker in a bioassay is similar to its K_d.

Eq. (1) shows that the τ_{on} of two compounds can be very different even if their k_{+1} is similar, provided that their k_{-1}, and hence their K_d, is very different (see Figure 2e).

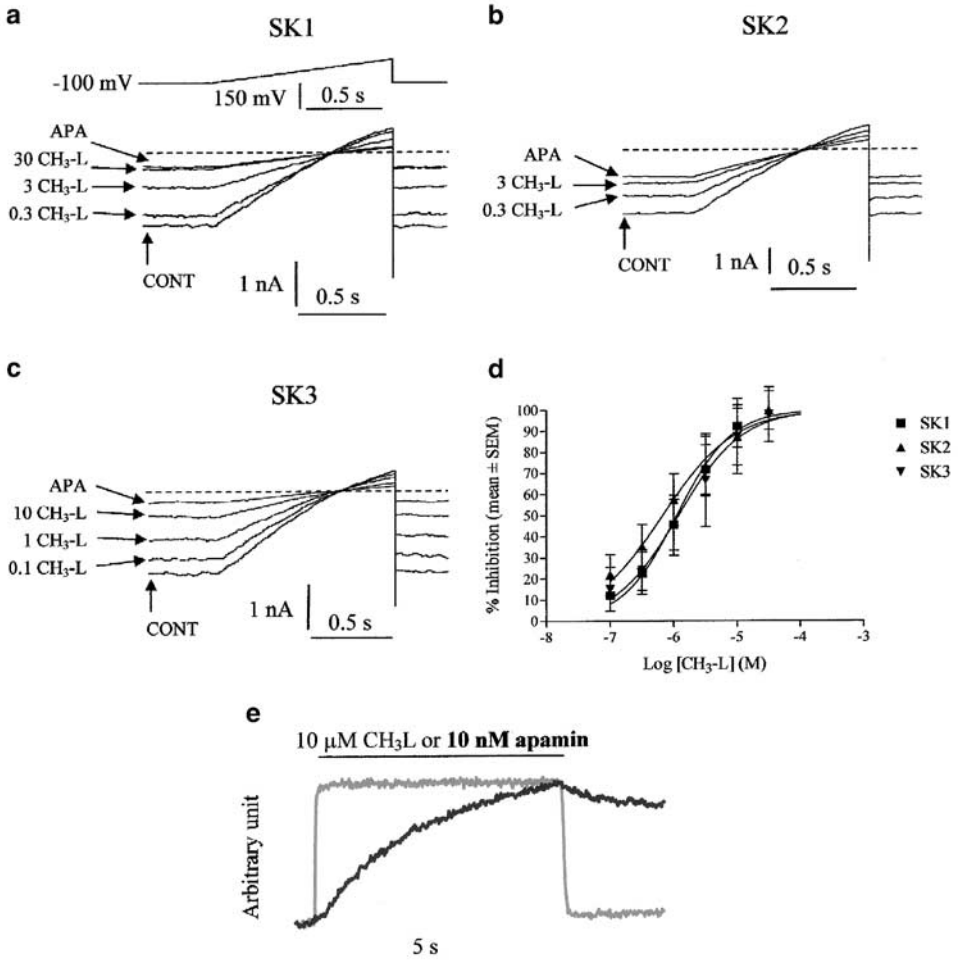

Figure 2 Blockade of SK currents by methyl-laudanosine in transfected cell lines. (a), (b) and (c) IV ramps from −100 to +50 mV obtained in control conditions and in the presence of increasing concentrations of methyl-laudanosine (in μM) and of 300 nM apamin (APA) in a SK1, a SK2 and a SK3 cell, respectively. For clarity, only currents measured in the presence of 2–3 concentrations of methyl-laudanosine are shown. The dashed line indicates the 0 current level. (d) Concentration–response curves obtained with methyl-laudanosine at the holding potential (−100 mV) in cells transfected with SK1, SK2 or SK3 channels. The maximal effect was defined as the amplitude of the current blocked by 300 nM apamin. (e) Comparison of the kinetics of wash-in and wash-out of the effects of 10 μM methyl-laudanosine (gray trace) and 10 nM apamin (black trace) in a SK3 cell. The traces were scaled to allow a better comparison. The holding potential was −80 mV.

Numerical values are expressed as means ± s.e.m. Statistical analysis was performed using Student's *t*-test or a one-way ANOVA followed by Dunnett's *post hoc* test when distributions were normal. Otherwise, a non–parametric test (Mann–Whitney or Kruskal–Wallis test) was used. The level of significance was set at $P<0.05$.

Drugs and solutions

Apamin was purchased from ICN Laboratories, or Sigma and stock solutions (333 ×) were prepared in distilled water. Methyl-laudanosine and methyl-noscapine were synthesized and isolated as iodide salts in our laboratory. Their stock solutions (at least 100 ×) were also prepared in distilled water. All other compounds were obtained from Sigma, except tetrodotoxin (ICN, Aurora, OH, U.S.A.) and SR95531, which was a generous gift from Sanofi-Synthelabo (Paris, France).

Noscapine and clotrimazole were first dissolved in DMSO. Final solutions contained less than 1% DMSO and all other solutions in experiments using these compounds contained the same final concentration of DMSO.

Results

Methyl-laudanosine equipotently blocks SK1, SK2 and SK3 currents in cell lines

In a high K^+ solution and using an intracellular solution yielding 1 μM free internal Ca^{2+}, voltage ramps (−100 to +40 mV) resulted in inwardly rectifying currents as shown in Figure 2. The amplitude of the inward current at −100 mV ranged from −1.2 to −8 nA. We measured the blocking potency of various concentrations of methyl-laudanosine (from 100 nM to 100 μM). As already mentioned, 300 nM apamin was used as a reference.

As can be seen in Figure 2, neither apamin nor methyl-laudanosine completely blocked recorded currents. This has also been reported by others (Dale *et al*., 2002). The fraction of inward current blocked at −100 mV in the three SK cell lines by 300 nM apamin ranged between 40 and 90%. The exact nature of the apamin-resistant current is unknown (see Discussion).

Methyl-laudanosine inhibited SK1, SK2 and SK3 currents in a concentration-dependent manner. The IC_{50} of methyl-laudanosine was 1.2 ± 0.2, 0.8 ± 0.1 and 1.8 ± 0.6 μM for SK1, SK2 and SK3 currents, respectively ($n=11$, 11 and 12) (Figure 2). These values were not significantly different from each other ($P=0.18$, Kruskal–Wallis test). Hill coefficients were 1.1 ± 0.1, 0.8 ± 0.1 and 0.9 ± 0.1 in the three cell lines, respectively.

A concentration of 100 μM methyl-laudanosine inhibited a slightly more important fraction of the inward current measured at −100 mV than 300 nM apamin (109 ± 2, 111 ± 2 and 106 ± 3% of the current blocked by apamin, for SK1, SK2 and SK3 cells, respectively), but the difference was not significant for any cell line (Student's *t*-test). The blockade of SK currents by methyl-laudanosine did not appear to be voltage-dependent in the range of voltages which were examined.

On the other hand, methyl-laudanosine did not block IK currents. At a concentration of 100 μM, it inhibited only 7 ± 4% of the clotrimazole-sensitive current (NS, Student's *t*-test) in IK cells ($n=7$, data not shown).

The effect of methyl-laudanosine is quickly reversible

While performing the concentration–response experiments, we noticed that the effect of methyl-laudanosine was much more quickly reversible than the one of apamin. This was further characterized in the SK3 cells. The results are summarized in Table 1. Concerning methyl-laudanosine, we only measured off-rates using three different concentrations of the drug. It can be seen that these rates were only 3–4 times greater than the exchange rate of our system (15 ms, see Methods), suggesting that our estimation is not completely accurate (see Discussion). However, off-rates measured for the three different concentrations of the compound were not significantly different from each other ($P=0.771$, one-way ANOVA), suggesting that our values are not too much distorted. These experiments allowed us to estimate that the k_{-1} for this compound is $\geqslant 20\,s^{-1}$ (Figure 2e). Because off-rates are slower than on-rates, we did not attempt to estimate the latter, since our observations would be even less accurate. On the other hand, both on- and off-rates were much slower for apamin, whose k_{-1} was found to be around $0.028\,s^{-1}$ (Figure 2e, Table 1).

Methyl-laudanosine blocks apamin-sensitive AHPs in 5-HT and NA neurones, but induces a steady non-SK-mediated hyperpolarization in 5-HT neurones

Figure 3 shows the blockade by methyl-laudanosine of the apamin-sensitive AHP in 5-HT and NA neurones. An effect

Table 1 Kinetic aspects of SK3 current blockade by methyl-laudanosine and apamin

Compound	*Concentration*	τ_{on} (s)	τ_{off} (s)	*Steady-state* IC_{50}
Methyl-laudanosine	1 μM	ND	0.051 ± 0.012[a] (6)	1.8 ± 0.6 μM (12)
	10 μM	ND	0.062 ± 0.010[a] (5)	
	100 μM	ND	0.056 ± 0.010[a] (7)	
Apamin	10 nM	1.945* (8)	29.678 ± 6.377[b] (8)	0.45 ± 0.05 nM (2)
	100 nM	0.695* (8)	42.375 ± 6.208[b] (8)	

ND = not determined (because the event was too fast as compared with the speed of solution exchange of our set-up).
Values within parentheses represent the number of experiments.
*Only the median value is given because the normality test failed. The two values are significantly different from each other ($P<0.001$, Mann–Whitney rank-sum test).
[a,b]These values are not significantly different from each other.

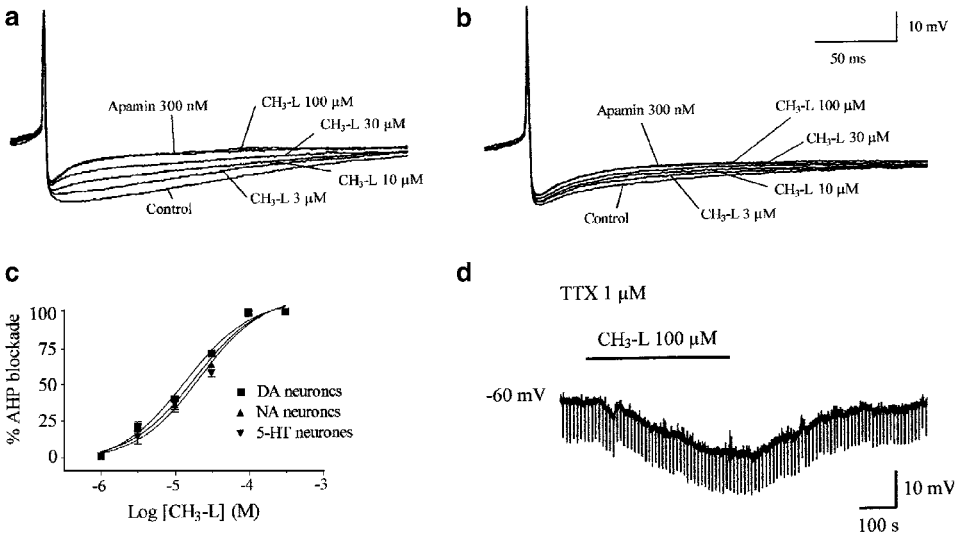

Figure 3 Methyl-laudanosine blocks apamin-sensitive AHPs in brainstem monoaminergic neurones, but has a nonspecific effect in 5-HT neurones. (a) Effect of increasing concentrations of methyl-laudanosine on the AHP of a 5-HT neurone. Apamin and 100 μM methyl-laudanosine blocked about 80% of the mAHP. (b) Same experiment on a NA neurone of the locus coeruleus. It can be seen that the fast AHP is unaffected, but that a major part ($\sim$60%) of the mAHP is blocked both by apamin and by the highest concentration of methyl-laudanosine. (c) Concentration–response curves for the blockade of AHPs in the three monoamine cell groups (the concentration–response curve in DA neurones was taken with permission from Scuvée-Moreau *et al*., 2002). The maximal effect was the surface area of the AHP which was blocked by 300 nM apamin. (d) One experiment showing that methyl-laudanosine directly hyperpolarizes 5-HT neurones, but does not modify their input resistance. The drug was superfused during the period indicated by the bar -20 pA current pulses (1 s in duration) were injected throughout the experiment. Tetrodotoxin was present throughout the experiment.

similar to the one observed upon application of 300 nM apamin was always obtained in the presence of 100 μM methyl-laudanosine. Concentration–response curves in the three monoamine cell groups are shown in Figure 3c. Curve fitting yielded IC_{50} values of 21 ± 2 and 19 ± 2 μM in 5-HT and NA neurones, respectively ($n=6$ in both cases). Hill coefficients were 1.1 ± 0.1 and 1.15 ± 0.3, respectively. In these experiments, increasingly positive current had to be injected in order to maintain the same firing rate in 5-HT neurones, suggesting a tonic hyperpolarizing effect of methyl-laudanosine. This was confirmed in an additional set of experiments (Figure 3d). In the presence of 1 μM TTX, 100 μM methyl-laudanosine induced a hyperpolarization of 9 ± 1 mV from -65 mV. This effect was antagonized by 1–10 μM atropine (not shown), but with a quite variable potency. Indeed, the amplitude of the blockade could be 0% ($n=1$), $\sim$50% ($n=3$) or 90% ($n=1$). This suggests a partial implication of muscarinic receptors, in agreement with previous binding data (Scuvée-Moreau *et al*., 2002). The reason for such an inconsistent effect of atropine is unknown. Apamin never produced such a hyperpolarization. On the other hand, methyl-laudanosine had no significant effect on action potential parameters (amplitude and duration at mid-height) of any of these cell types (Table 2).

Methyl-noscapine blocks SK1, SK2 and SK3 currents in cell lines

For these experiments, we used 100 μM methyl-laudanosine as the reference, since this concentration blocked all SK channels to the same extent as 300 nM apamin. Figure 4 shows the ability of methyl-noscapine to block the various SK currents. Its IC_{50} was 5.9 ± 0.9, 5.6 ± 0.8 and 3.9 ± 0.8 μM for SK1, SK2 and SK3 currents, respectively ($n=11$, 11 and 12). These values were not significantly different from each other ($P=0.06$, Kruskal–Wallis test). The compound was significantly less potent than methyl-laudanosine on SK1 and SK2 ($P<0.001$, Student's t-test), but not on SK3 currents ($P=0.07$). Hill coefficients were 0.8 ± 0.1 in the three cell lines. The effect of methyl-noscapine was very quickly reversible in all cell lines.

On the other hand, methyl-noscapine failed to significantly block IK currents. At a concentration of 100 μM, it blocked the clotrimazole-sensitive current by only 10 ± 7% (NS, Student's t-test) ($n=4$).

Finally, the corresponding tertiary amine, noscapine, which is used in some countries as an antitussive agent, had very little effect on SK currents. At a concentration of 100 μM, it inhibited only 9 ± 8, 22 ± 10 and 19 ± 3% of the current blocked by 100 μM methyl-laudanosine in SK1, SK2 and SK3 cells, respectively ($n=4$, 5 and 5). Its effect was only significant in SK3 cells ($P<0.001$, Student's t-test).

Methyl-noscapine specifically blocks apamin-sensitive AHPs in DA, 5-HT and NA neurones

As shown in Figure 5, methyl-noscapine blocked mAHPs in all monoaminergic cell groups, albeit with overall less potency than methyl-laudanosine. Its IC_{50} in DA, 5-HT and NA neurones was 42 ± 4, 37 ± 8 and 29 ± 3 μM ($n=6$), respectively. These values were significantly different from those obtained with methyl-laudanosine in DA ($P<0.001$, Student's t-test) and NA cells ($P<0.05$), but not in 5-HT neurones ($P=0.08$). Hill coefficients were 1 ± 0.2, 0.98 ± 0.03 and 1.04 ± 0.05 in the three cell types, respectively. An effect equal to the one of

Table 2 Lack of effect of 100 μM methyl-laudanosine and 300 μM methyl-noscapine on various electrophysiological parameters of monoaminergic neurones

	Methyl-laudanosine			*Methyl-noscapine*		
	Control	*Drug*	n	*Control*	*Drug*	n
DA neurones						
AP amplitude (mV)	50±2.5[a]	50±2.5[a]	4	55.8±2.0	55.6±2.0	8
AP duration (ms)	1.3±0.2[a]	1.3±0.2[a]	4	1.1±0.1	1.1±0.1	8
R cell (MΩ)	231±22[a]	231±22[a]	4	145±17	147±17	6
I_h[b]	10±0.6[a]	10±0.6[a]	4	11.5±1.0	12±0.8	6
5-HT neurones						
AP amplitude (mV)	59±2.1	58±2.6	6	58±1.3	58±1.2	7
AP duration (ms)	1.5±0.07	1.5±0.07	6	1.2±0.02	1.2±0.03	7
R cell (MΩ)	165±15	Not tested	6	289±37	291±37	7
NA neurones						
AP amplitude (mV)	59.8±0.9	59.8±0.9	6	57.9±1.4	57.5±1.7	8
AP duration (ms)	1.1±0.06	1.1±0.06	6	1.1±0.04	1.1±0.03	8
R cell (MΩ)	108±16	Not tested	6	119±25	120±25	7

[a]Data from Scuvée-Moreau *et al.* (2002).
[b]A putative effect of the drug on the amplitude of this current was evaluated in current-clamp by measuring the amplitude of the depolarizing sag produced by the activation of the current during a 100–150 pA hyperpolarizing current injection from −60 mV.
Values represent the number of experiments.

300 nM apamin was reached in the three cell types with 300 μM methyl-noscapine. Contrary to methyl-laudanosine, methyl-noscapine (300 μM) did not significantly affect the membrane potential of any of these neurones, including the 5-HT cells ($n=8$, 6 and 3 in 5-HT, DA and NA neurones) (data not shown). Furthermore, this compound had no effect on other electrophysiological parameters of these cell types (Table 2).

Methyl-noscapine (100–300 μM) was devoid of $GABA_A$ antagonism: indeed, it did not modify the decrease in input resistance induced by muscimol (3 μM), while the $GABA_A$ antagonist SR95531 (10 μM) completely reversed the effect of muscimol ($n=3$) (data not shown).

Methyl-noscapine has the same effect as apamin on the excitability of monoaminergic neurones

In order to explore the consequences of AHP blockade on the excitability of the three types of monoaminergic neurones, the effect of maximally active concentrations of methyl-noscapine and apamin on the number of spikes induced by long depolarizing pulses of increasing intensities was explored. As shown in Figure 6, both methyl-noscapine (300 μM) and apamin (300 nM) induced a significant increase in the excitability of midbrain dopaminergic ($n=5$) and locus coeruleus noradrenergic ($n=6$) neurons. Dorsal raphe serotonergic neurons had a more variable response to the application of AHP blockers with only four out of seven cells showing an increase in their excitability. There was no apparent difference in the electrophysiological characteristics of the two types of cells.

Discussion

The aim of this work was to characterize the blockade of SK channels by methyl-laudanosine and methyl-noscapine. Our cell line experiments demonstrate that both compounds are able to block all of the apamin-sensitive current in the three SK cell lines whereas they do not affect IK currents. We consistently observed that a fraction of the current in the SK cell lines was not blocked by any SK channel blocker. The reason for this is unclear. Others have suggested that a fraction of SK current is not sensitive to apamin in this kind of preparation (Dale *et al.*, 2002). In support of this conclusion, these authors showed that the apamin-resistant current behaved like a SK current in terms of Ca^{2+} dependence, relative permeation of monovalent cations, resistance to blockers of other channels and sensitivity to cyproheptadine. Because we did not perform these experiments, we cannot draw conclusions about the nature of the apamin-insensitive current, which could be a blocker-resistant SK current or another endogenous current of the cells. The latter hypothesis is rather unlikely, however, because untransfected cells had currents that were much smaller than the apamin-insensitive current in the transfected cells. Methyl-laudanosine tended to block more current than apamin in the three cell lines. However, the difference was not significant overall. Because methyl-laudanosine did not alter any other electrophysiological parameter than the mAHP in slices (except in 5-HT neurones), we do not think that the observation in cell lines is due to the blockade of other channels than the SK channels.

Both methyl-laudanosine and methyl-noscapine blocked mAHPs in slices with IC_{50}s that were about an order of magnitude larger than their IC_{50}s in the cell lines. It is possible that the pharmacology of cloned channels is different from the one of native channels, particularly if the latter turns out to be heteromeric. Such a difference between heteromeric and homomeric channels has been demonstrated in heterologous expression systems (Benton *et al.*, 2003; Monaghan *et al.*, 2004). Another possible explanation for this difference is that the access of both compounds to the channels was more difficult in the slices. It could be argued that the same holds for

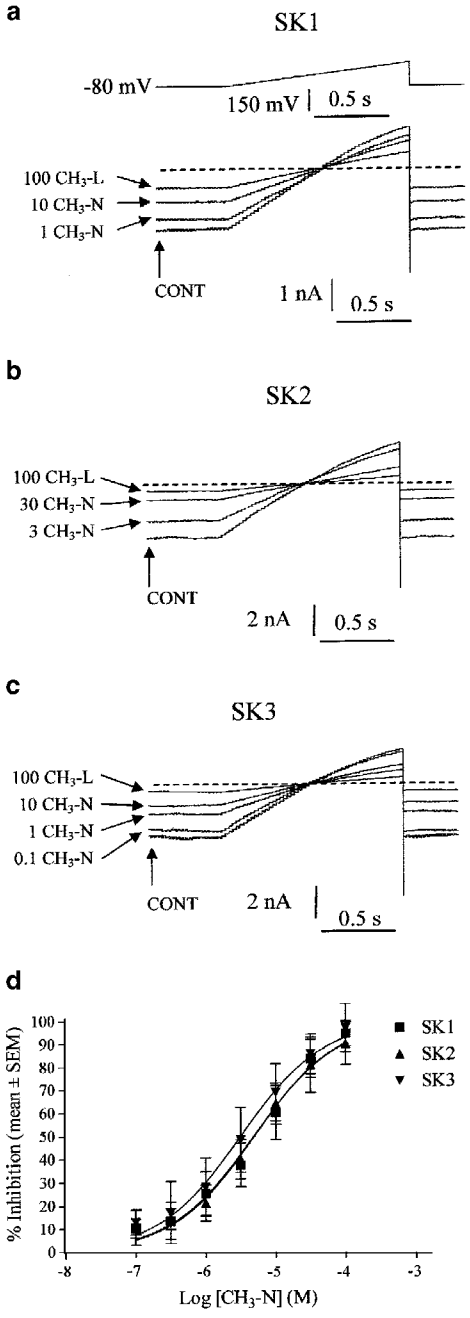

Figure 4 Methyl-noscapine blocks SK1, SK2 and SK3 currents in transfected cell lines (IV ramps from −80 to +70 mV). (a), (b) and (c) Representative experiment performed in a SK1, a SK2 and a SK3 cell, respectively. The dashed line indicates the 0 current level. All concentrations are in μM. (d) Concentration–response curves for methyl-noscapine in the three cell lines at the holding potential (−80 mV). The maximal effect was defined as the current blocked by 100 μM methyl-laudanosine.

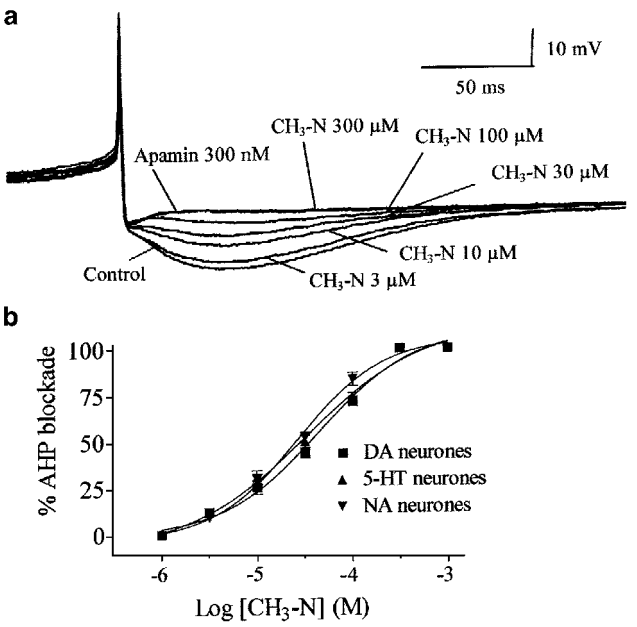

Figure 5 Methyl-noscapine blocks apamin-sensitive AHPs in brainstem monoaminergic neurones. (a) Effect of increasing concentrations of methyl-noscapine on the AHP of a DA neurone. (b) Concentration–response curves for the blockade of AHPs by methyl-noscapine in the three monoamine cell groups. The maximal effect was defined as in Figure 3.

apamin; however, the toxin was used at a largely supramaximal concentration which is very likely to produce a full blockade in both preparations.

Our experiments show that methyl-laudanosine equipotently blocks all SK channel subtypes, but that its use as a SK blocker may be problematic in some neurones because of its non-SK-mediated effect that we have observed only in 5-HT neurones so far. Indeed, it was not found in DA, NA or hippocampal pyramidal neurones (this paper and Scuvée-Moreau *et al*., 2002). On the other hand, methyl-noscapine appears to be another valuable tool to probe the physiological roles of SK channels. Indeed, we did not observe any significant non-SK channel-mediated effects with this compound and our data show that it should affect neither conventional voltage-dependent Na^+, K^+ and I_h channels, nor $GABA_A$ receptors. Interestingly, we recently found that the non-SK action of methyl-laudanosine (which is a racemic mixture) is due to its (−) enantiomer (Scuvée-Moreau *et al*., 2004). Therefore, the (+) enantiomer of methyl-laudanosine may also represent a good pharmacological tool.

Methyl-noscapine modulated the excitability of monoaminergic neurones very similarly to apamin. Although, as expected, an increase in excitability was observed in all dopaminergic and noradrenergic neurones which were tested, this was not the case in serotonergic neurones. In 40% of these, no increased excitability was observed, neither with methyl-noscapine nor with apamin. The explanation for this observation is unknown and deserves further investigation.

Our experiments do not allow to determine what effect SK channel blockade will have on the firing pattern and firing rate of brain monoaminergic neurons under physiological conditions. We are currently addressing this question by using single cell recordings and iontophoresis of methyl-laudanosine and methyl-noscapine in anesthetized rats (Seutin *et al*., 2002; Waroux *et al*., in preparation).

One of the main interests of both methyl-laudanosine and methyl-noscapine probably lies in their quickly reversible effect. We attempted to quantify this for methyl-laudanosine in SK3 cells. The accuracy of our measurements was unfortunately limited by the speed of solution exchange of our fast superfusion system. Indeed, this speed was less than 10 times

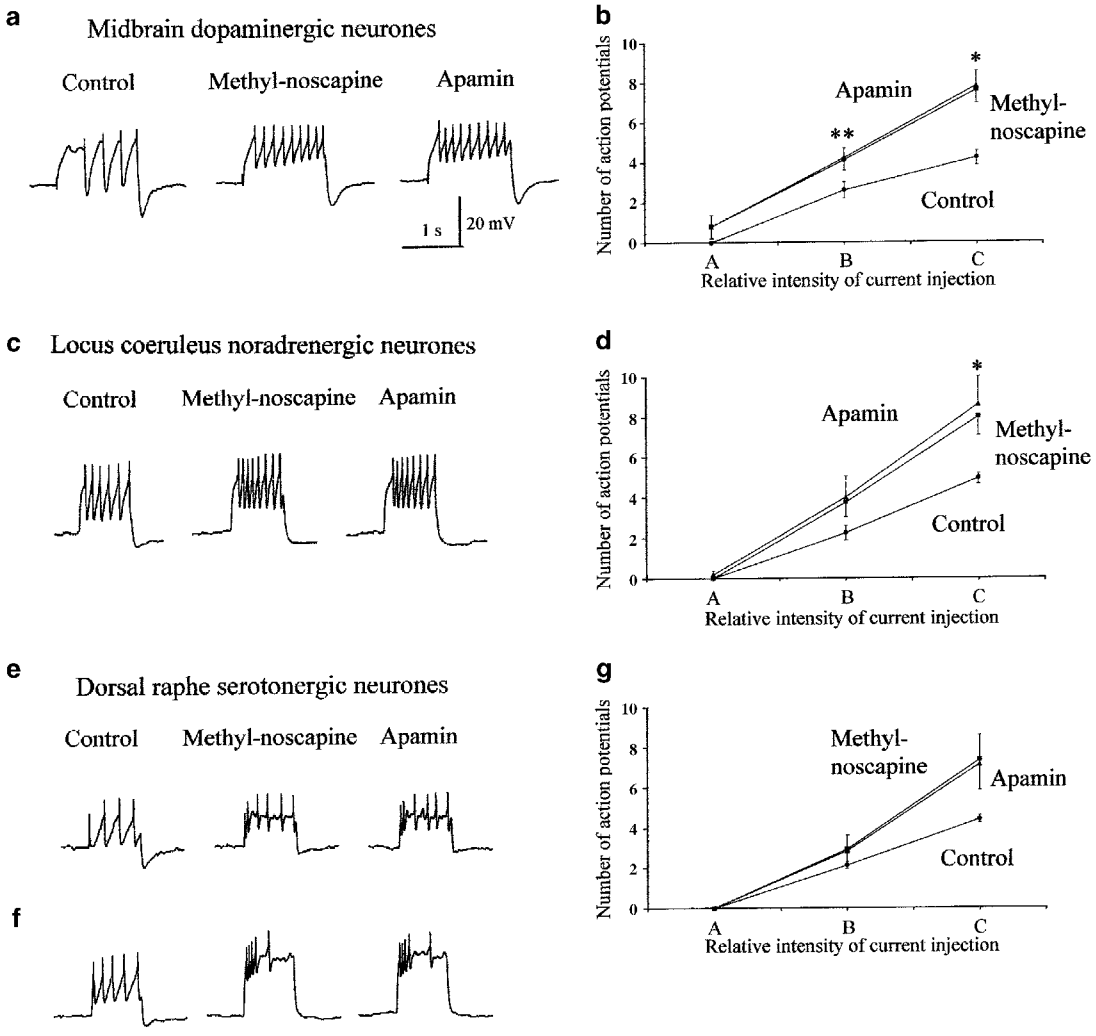

Figure 6 Effect of methyl-noscapine and apamin on cell excitability. a, c, e and f: Action potentials (truncated in the figure) were elicited by 400 pA (a) and 150 pA (c, e, f) depolarizing current pulses before and during the application of 300 μM methyl-noscapine followed by 300 nM apamin; membrane potential was set at -65 mV by a continuous current injection of -270 pA (a) or -170 pA (c) or -10 pA (e). b, d and g: The number of action potentials elicited by the pulses are plotted against the level of intensity of the injected current. Methyl-noscapine and apamin induced a similar significant increase in the number of action potentials evoked ($*P<0.05$; $**P<0.005$) in dopaminergic and noradrenergic neurons. The variability of the effect on DR neurons is illustrated by the examples given in (e) and (f). Values are means of five (b), six (d) and seven (g) experiments. Note that SK channel blockade had variable consequences on the AHP observed at the end of the pulse in the various cell types.

faster than the τ_{off} of methyl-laudanosine. Therefore, our measurements probably overestimate the real τ_{off}. Because the measured τ_{off} values were roughly similar for three concentrations of the drug, as expected from the theory, we can nevertheless conclude that the real value is not far from our estimate, which was $\sim$50 ms. This would yield a k_{-1} value $\geqslant 20\,s^{-1}$ (using the equation $k_{-1}=1/\tau_{off}$). Assuming that the IC_{50} is close to the K_d for an ion channel blocker (see Methods), the k_{+1} of methyl-laudanosine should be close to $1.1\times10^7\,M^{-1}\,s^{-1}$. For apamin, we found that the k_{-1} is $\sim 0.028\,s^{-1}$ (using the same equation and the mean of the values reported in Table 1 for τ_{off}) and the k_{+1} lies between 1.4 and 6 $10^7\,M^{-1}\,s^{-1}$ (depending on the method used to calculate it). Thus, the difference in affinity between the two compounds is essentially due to a major difference between their k_{-1}.

The mean residency time of methyl-laudanosine and apamin should be $\leqslant$50 ms and $\sim$36 s, respectively. Methyl-laudanosine can therefore be considered as an intermediate drug and apamin as a slow drug, according to Hille (2001). This conclusion is consistent with recent single-channel inside-out experiments that we have performed on transiently transfected SK channels. A flickering block was observed with methyl-laudanosine, whereas a much slower block was found for apamin (Seutin, Mizielinska and Marrion, unpublished observations).

We believe that the interest of rapidly dissociating SK blockers may be double. First, they may be a valuable addition to the pharmacological armamentarium of SK blockers, because their effect will be much more readily reversible than the one of high-affinity blockers. Indeed, wash-out of apamin effects are rarely reported in brain slice experiments. In addition, because of their quaternary nature, methyl-laudanosine and methyl-noscapine are highly water-soluble and can be used for iontophoretic application *in vivo* (see above).

Secondly, with the perspective of developing SK channel blockers with therapeutic applications in the CNS, low-affinity SK blockers may be more tolerable than high-affinity compounds. Apamin has clearly a very narrow therapeutic window. Several toxic effects, including convulsions and even neurodegeneration, have been reported (Habermann & Cheng-Raude, 1975; Mourre *et al*., 1997; van der Staay *et al*., 1999). It will be important to evaluate this aspect for low-affinity compounds. Although very speculative, a parallel might be drawn with Na^+ channel blockers. All compounds in clinical use, such as phenytoin and carbamazepine, are low-affinity compounds whereas the high-affinity toxin tetrodotoxin is extremely toxic. Similarly, in the field of D2 receptor blockers, low affinity blockers such as clozapine induce significantly less extrapyramidal side effects than potent antagonists such as haloperidol (Kapur & Seeman, 2001), although it is clear that other factors may contribute in this case.

The compounds described in this paper will probably not provide CNS therapeutic effects because of their quaternary nature. Therefore, in parallel with the discovery of water-soluble pharmacological tools, we are developing basic compounds which are more likely to cross the blood–brain barrier after systemic administration. Further electrophysiological and behavioral experiments will be needed to determine their potential interest in therapeutics. Another important step will also be to develop subtype-specific blockers.

In conclusion, methyl-laudanosine and methyl-noscapine are medium potency, quickly dissociating SK channel blockers with no significant selectivity for one SK channel subtype. Methyl-noscapine appears to be more selective *versus* non-SK targets.

J.-F.L. is a Senior Research Associate of the 'Fonds National pour la Recherche Scientifique' (FNRS). F.G.-L. was a Research Fellow of the FNRS. A.G. is a Research Fellow of the 'Fonds pour la Formation à la Recherche dans l'Industrie et l'Agriculture' (FRIA). M. S. and D. D. acknowledge support from the Wellcome Trust. M.S. is a Wellcome Trust Senior Research Fellow. This work was supported by Grant n° 9.4560.03 from the FNRS and by a grant from the 'Fonds Facultaire de la Recherche' of the Faculty of Medicine of the University of Liège (V.S., J.S.-M. and J.-F.L.). We wish to thank the editor and referees of an earlier version of this manuscript for their constructive criticisms and valuable suggestions. We also thank Laurent Massotte and Nicole Delvaux for their help with the iconography.

References

ABIA, A., LOBATON, C., MORENO, A. & GARCIA-SANCHO, J. (1986). *Leiurus quinquestriatus* venom inhibits different kinds of Ca^{2+}-dependent K^+ channels. *Biochim. Biophys. Acta – Biomembranes,* **856,** 403–407.

ADEAGBO, A.S.O. (1999). 1-Ethyl-2-benzimidazolinone stimulates endothelial K_{ca} channels and nitric oxide formation in rat mesenteric vessels. *Eur. J. Pharmacol.,* **379,** 151–159.

ANTONARAKIS, SE., BLOUIN, J.L., LASSETER, V.K., GEHRIG, C., RADHAKRISHNA, U., NESTADT, G., HOUSMAN, D.E., KAZAZIAN, H.H., KALMAN, K., GUTMAN, G., FANTINO, E., CHANDY, K.G., GARGUS, J.J. & PULVER, A.E. (1999). Lack of linkage or association between schizophrenia and the polymorphic trinucleotide repeat within the KCNN3 gene on chromosome 1q21. *Am. J. Med. Genet.,* **88,** 348–351.

ARMSTRONG, C.M. & GILLY, W.F. (1992). Access resistance and space clamp problems associated with whole-cell patch clamping. In: *Methods in Enzymology,* ed. Rudy, B. & Iverson, L.E. Vol. 207, pp. 100–122. San Diego: Academic Press.

BENTON, D.C., MONAGHAN, A.S., HOSSEINI, R., BAHIA, P.K., HAYLETT, D.G. & MOSS, G.W. (2003). Small conductance Ca^{2+}-activated K^+ channels formed by the expression of rat SK1 and SK2 genes in HEK 293 cells. *J. Physiol.,* **553,** 13–19.

BONNET-BRILHAULT, F., LAURENT, C., CAMPION, C., CAMPION, D., THIBAUT, F., LAFARQUE, C., CHARBONNIER, F., DELEUZE, J.F., MENARD, J.F., JAY, M., PETIT, M., FREBOURG, T. & MALLET, J. (1999). No evidence for involvement of KCNN3 (hSKCa3) potassium channel gene in familial and isolated cases of schizophrenia. *Eur. J. Hum. Genet.,* **7,** 247–250.

CAMPOS ROSA, J., GALANAKIS, D., PIERGENTILI, A., BHANDARI, K., GANELLIN, C.R., DUNN, P.M. & JENKINSON, D.H. (2000). Synthesis, molecular modeling, and pharmacological testing of bis-quinolinium cyclophanes: potent, non-peptidic blockers of the apamin-sensitive Ca^{2+}-activated K^+ channel. *J. Med. Chem.,* **43,** 420–431.

CAO, Y.J., DREIXLER, J.C., ROIZEN, J.D., ROBERTS, M.T. & HOUAMED, K.M. (2001). Modulation of recombinant small-conductance Ca^{2+}-activated K^+ channels by the muscle relaxant chlorzoxazone and structurally related compounds. *J. Pharmacol. Exp. Ther.,* **296,** 683–689.

CAO, Y.J. & HOUAMED, K.M. (1999). Activation of recombinant human SK4 channels by metal cations. *FEBS Lett.,* **446,** 137–141.

CHEN, H., KIM, L.A., RAJAN, S., XU, S. & GOLDSTEIN, S.A.N. (2003). Charybdotoxin binding in the I_{Ks} pore demonstrates two MinK subunits in each channel complex. *Neuron,* **40,** 15–23.

CHICCHI, G.G., GIMENEZ-GALLEGO, G., BER, E., GARCIA, M.L., WINQUIST, R. & CASCIERI, M.A. (1988). Purification and characterization of a unique, potent inhibitor of apamin binding from *Leiurus quinquestriatus hebraeus* venom. *J. Biol. Chem.,* **263,** 10192–10197.

CINGOLANI, L.A., GYMNOPOULOS, M., BOCCACCIO, A., STOCKER, M. & PEDARZANI, P. (2002). Developmental regulation of small-conductance Ca^{2+}-activated K^+ channel expression and function in rat Purkinje neurons. *J. Neurosci.,* **22,** 4456–4467.

DALE, T.J., CRYAN, J.E., CHEN, M.X. & TREZISE, D.J. (2002). Partial apamin sensitivity of human small conductance Ca^{2+}-activated K^+ channels stably expressed in Chinese hamster ovary cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **366,** 470–477.

DROR, V., SHAMIR, E., GHANSHANI, S., KIMHI, R., SWARTZ, M., BARAK, Y., WEIZMAN, R., AVIVI, L., LITMANOVITCH, T., FANTINO, E., KALMAN, K., JONES, E.G., CHANDY, K.G., GARGUS, J.J., GUTMAN, G.A. & NAVON, R. (1999). *hKCa3/KCNN3* potassium channel gene: association of longer CAG repeats with schizophrenia in Israeli Ashkenazi Jews, expression in human tissues and localization to chromosome 1q21. *Mol. Psychiatry,* **4,** 254–260.

FINLAYSON, K., MCLUCKIE, J., HERN, J., ARAMORI, I., OLVERMAN, H.J. & KELLY, J.S. (2001). Characterisation of [^{125}I]-apamin binding sites in rat brain membranes and HEK293 cells transfected with SK channel subtypes. *Neuropharmacology,* **41,** 341–350.

GUTMAN, G.A., CHANDY, G., ADELMAN, J.P., AIYAR, J., BAYLISS, D.A., CLAPHAM, D.E., COVARRIUBIAS, M., DESIR, G.V., FURUICHI, K., GANETZKY, B., GARCIA, M.L., GRISSMER, S., JAN, L.Y., KARSHIN, A., KIM, D., KUPERSCHMIDT, S., KURACHI, Y., LAZDUNSKI, M., LESAGE, F., LESTER, H.A., MCKINNON, D., NICHOLS, G., O'KELLY, I., ROBBINS, J., ROBERTSON, A., RUDY, B., SANGUINETTI, M., SEINO, S., STUEHMER, W., TAMKUN, M.M., VANDENBERG, C.A., WEI, A., WULFF, H. & WYMORE, R.S. (2003). International Union of Pharmacology, XLI. Compendium of voltage-gated ion channels: potassium channels. *Pharmacol. Rev.,* **55,** 583–586.

HABERMANN, E. & CHENG-RAUDE, D. (1975). Central neurotoxicity of apamin, crotamin, phospholipase A and α-amanitin. *Toxicon,* **13,** 465–473.

HILLE, B. (2001). *Ion Channels of Excitable Membranes,* 3rd edn. Sunderland, MA, U.S.A.: Sinauer Associates, Inc..

IKONEN, S. & RIEKKINEN Jr, P. (1999). Effects of apamin on memory processing of hippocampal-lesioned mice. *Eur. J. Pharmacol.,* **382,** 151–156.

INNIS, R.B., NESTLER, E.J. & AGHAJANIAN, G.K. (1988). Evidence for G protein mediation of serotonin- and GABAB-induced hyperpolarization of rat dorsal raphe neurons. *Brain Res.,* **459,** 27–36.

ISHII, T.M., MAYLIE, J. & ADELMAN, J.P. (1997). Determinants of apamin and d-tubocurarine block in SK potassium channels. *J. Biol. Chem.,* **272,** 23195–23200.

JENSEN, B.S., STROBAEK, D., CHRISTOPHERSEN, P., JORGENSEN, T.D., HANSEN, C., SILAHTAROGLU, A., OLESEN, S.P. & AHRING, P.K. (1998). Characterization of the cloned human intermediate-conductance Ca^{2+}-activated K^+ channel. *Am. J. Physiol. Cell Physiol.,* **275,** C848–C856.

KAPUR, S. & SEEMAN, P. (2001). Does fast dissociation from the dopamine D(2) receptor explain the action of atypical antipsychotics ?: a new hypothesis. *Am. J. Psychiatry,* **158,** 360–369.

KÖHLEZ, M., HIRSCHBERG, B., BOND, C.T., KINZIE, J.M., MARRION, N.V., MAYLIE, J. & ADELMAN, J.P. (1996). Small-conductance, calcium-activated potassium channels from mammalian brain. *Science,* **273,** 1709–1714.

LATORRE, R., OBERHAUSER, A., LABARCA, P. & ALVAREZ, O. (1989). Varieties of Ca^{2+}-activated K^+ channels. *Annu. Rev. Physiol.,* **51,** 385–399.

LEGROS, C., OUGHUIDENI, R., DARBON, H., ROCHAT, H., BOUGIS, P.E. & MARTIN-EAUCLAIRE, M.F. (1996). Characterization of a new peptide from *Tityus serrulatus* scorpion venom which is a ligand of the apamin binding site. *FEBS Lett.,* **390,** 81–84.

LIÉGEOIS, J.-F., MERCIER, F., GRAULICH, A., GRAULICH-LORGE, F., SCUVEE-MOREAU, J. & SEUTIN, V. (2003). Modulation of small conductance calcium-activated potassium (SK) channels: a new challenge in medicinal chemistry. *Curr. Med. Chem.,* **10,** 625–647.

MILLER, M., RAUER, H., TOMITA, H., GARGUS, J.J., GUTMAN, G.A., CAHALAN, G.A. & CHANDY, K.G. (2001). Nuclear localization and dominant-negative suppression by a mutant SKCa3 *N*-terminal channel fragment identified in a patient with schizophrenia. *J. Biol. Chem.,* **276,** 27753–27756.

MONAGHAN, A.S., BENTON, D.C., BAHIA, P.K., HOSSEINI, R., SHAH, Y.A., HAYLETT, D.G. & MOSS, G.W. (2004). The SK3 subunit of small conductance Ca^{2+}-activated K^+ channels interacts with both SK1 and SK2 subunits in a heterologous expression system. *J. Biol. Chem.,* **279,** 1003–1009.

MOURRE, C., FOURNIER, C. & SOUMIREU-MOURAT, B. (1997). Apamin, a blocker of the calcium-activated potassium channel, induces neurodegeneration of Purkinje cells exclusively. *Brain Res.,* **778,** 405–408.

PAXINOS, G. & WATSON, C. (1986). *The Rat Brain in Stereotaxic Coordinates,* 2nd edn. San Diego, CA, U.S.A.: Academic Press.

PEDARZANI, P., D'HOEDT, D., DOORTY, K.B., WADSWORTH, J.D., JOSEPH, J.S., JEYASEELAN, K., KINI, R.M., GADRE, S.V., SAPATNEKAR, S.M., STOCKER, M. & STRONG, P.N. (2002). Tamapin, a venom peptide from the Indian scorpion (*Mesobuthus tamulus*) that targets small-conductance Ca^{2+}-activated K^+ channels and afterhyperpolarization currents in central neurons. *J. Biol. Chem.,* **277,** 46101–46109.

PEDARZANI, P., KULIK, A., MULLER, M., BALLANYI, K. & STOCKER, M. (2000). Molecular determinants of Ca^{2+}-dependent K^+ channel function in rat dorsal vagal neurones. *J. Physiol. (Lond.),* **527,** 283–290.

PEDARZANI, P., MOSBACHER, J., RIVARD, A., CINGOLANI, L.A., OLIVER, D., STOCKER, M., ADELMAN, J.P. & FAKLER, B. (2001). Control of electrical activity in central neurons by modulating the gating of small conductance Ca^{2+}-activated K^+ channels. *J. Biol. Chem.,* **276,** 9762–9769.

RITSNER, M., MODAI, I., ZIV, H., AMIR, S., HALPERIN, T., WEIZMAN, R. & NAVON, R. (2002). An association of CAG repeats at the KCNN3 locus with symptom dimensions of schizophrenia. *Biol. Psych.,* **51,** 788–794.

SAH, P. (1996). Ca^{2+}-activated K^+ currents in neurones: types, physiological roles and modulation. *Trends Neurosci.,* **19,** 150–154.

SAH, P. & FABER, E.S.L. (2002). Channels underlying neuronal calcium-activated potassium currents. *Prog. Neurobiol.,* **66,** 345–353.

SAILER, C.A., HU, H., KAUFMANN, W.A., TRIEB, M., SCHWARZER, C., STORM, J.F. & KNAUS, H.G. (2002). Regional differences in distribution and functional expression of small-conductance Ca^{2+}-activated K^+ channels in rat brain. *J. Neurosci.,* **22,** 9698–9707.

SCUVÉE-MOREAU, J., GRAULICH, A., LIEGEOIS, J.-F. & SEUTIN, V. (2004). Effect of the SK channel blocker *N*-methyl-laudanosine on brainstem monoaminergic neurons in slices: differences among its stereoisomers. *Soc. Neurosci. Abstr.,* (In press).

SCUVÉE-MOREAU, J., LIEGEOIS, J.-F., MASSOTTE, L. & SEUTIN, V. (2002). Methyl-laudanosine: a new pharmacological tool to investigate the function of small-conductance Ca^{2+}-activated K^+ channels. *J. Pharmacol. Exp. Ther.,* **302,** 1176–1183.

SEUTIN, V. & JOHNSON, S.W. (1999). Recent advances in the pharmacology of quaternary salts of bicuculline. *Trends Pharmacol. Sci.,* **20,** 268–270.

SEUTIN, V., MASSOTTE, L., LIEGEOIS, J.-F. & SCUVEE-MOREAU, J. (2002). Effect of a new SK channel blocker, methyl-laudanosine, on the firing pattern of rat midbrain dopaminergic neurons *in vivo*. *Soc. Neurosci. Abst.,* **28,** 340 .5.

SEUTIN, V., SCUVEE-MOREAU, J. & DRESSE, A. (1997). Evidence for a non-GABAergic action of quarternary salts of bicuculline on dopaminergic neurones. *Neuropharmacology,* **36,** 1653–1657.

SHAKKOTTAI, V.G., REGAYA, I., WULFF, H., FAJLOUN, Z., TOMITA, H., FATHALLAH, M., CAHALAN, M.D., GARGUS, J.J., SABATIER, J.-M. & CHANDY, K.G. (2001). Design and characterization of a highly selective peptide inhibitor of the small conductance calcium-activated K^+ channel, SkCa2. *J. Biol. Chem.,* **276,** 43145–43151.

STACKMAN, R.W., HAMMOND, R.S., LINARDATOS, E., GERLACH, A., MAYLIE, J., ADELMAN, J.P. & TZOUNOPOULOS, T. (2002). Small conductance Ca^{2+}-activated K^+ channels modulate synaptic plasticity and memory encoding. *J. Neurosci.,* **22,** 10163–10171.

STOCKER, M., HIRZEL, K., D'HOEDT, D. & PEDARZANI, P. (2004). Matching molecules to function: neuronal Ca^{2+}-activated K^+ channels and afterhyperpolarizations. *Toxicon,* **43,** 933–949.

STOCKER, M., KRAUSE, M. & REDARZANI, P. (1999). An apamin-sensitive Ca^{2+}-activated K^+ current in hippocampal pyramidal neurons. *Proc. Natl. Acad. Sci. U.S.A.,* **96,** 4662–4667.

STOCKER, M. & PEDARZANI, P. (2000). Differential distribution of three Ca^{2+}-activated K^+ channel subunits SK(1–3) in the adult rat central nervous system. *Mol. Cell. Neurosci.,* **15,** 476–493.

TSAI, M.T., SHAW, C.K., HSIAO, K.J. & CHEN, C.H. (1999). Genetic association study of a polymorphic CAG repeats array of calcium-activated potassium channel (KCNN3) gene and schizophrenia among the Chinese population from Taiwan. *Mol. Psych.,* **4,** 271–273.

VAN DER STAAY, F.J., FANELLI, R.J., BLOCKLAND, A. & SCHMIDT, B.H. (1999). Behavioral effects of apamin, a selective inhibitor of the SK_{Ca}-channel, in mice and rats. *Neurosci. Biobehav. Rev.,* **23,** 1087–1110.

VANDERMAELEN, C.P. & AGHAJANIAN, G.K. (1983). Electrophysiological and pharmacological characterization of serotonergic dorsal raphe neurons recorded extracellularly and intracellularly in rat brain slices. *Brain Res.,* **289,** 109–119.

WOLFART, J., NEUHOFF, H., FRANZ, O. & ROEPER, J. (2001). Differential expression of the small-conductance, calcium-activated potassium channel SK3 is critical for pacemaker control in dopaminergic midbrain neurons. *J. Neurosci.,* **21,** 3443–3456.

XIA, X.M., FAKLER, B., RIVARD, A., WAYMAN, G., JOHNSON-PAIS, T., KEEN, J.E., ISHII, T., HIRSCHBERG, B., BOND, C.T., LUTSENKO, S., MAYLIE, J. & ADELMAN, J.P. (1998). Mechanism of calcium gating in small-conductance calcium-activated potassium channels. *Nature,* **395,** 503–507.

ZERROUK, H., MANSUELLE, P., BENSLIMANE, A., ROCHAT, H. & MARTIN-EAUCLAIRE, M.F. (1993). Characterization of a new leiurotoxin I-like scorpion toxin PO5 from *Androctonus mauretanicus mauretanicus*. *FEBS Lett.,* **320,** 189–192.

(Received July 15, 2004
Revised August 6, 2004
Accepted August 19, 2004)