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Molecular correlates of the M-current in cultured rat hippocampal neurons

M. M. Shah, M. Mistry, S. J. Marsh, D. A. Brown and P. Delmas

Wellcome Laboratory for Molecular Pharmacology, Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, UK

M-type K^+ currents ($I_{K(M)}$) play a key role in regulating neuronal excitability. In sympathetic neurons, M-channels are thought to be composed of a heteromeric assembly of KCNQ2 and KCNQ3 K^+ channel subunits. Here, we have tried to identify the KCNQ subunits that are involved in the generation of $I_{K(M)}$ in hippocampal pyramidal neurons cultured from 5- to 7-day-old rats. RT-PCR of either CA1 or CA3 regions revealed the presence of KCNQ2, KCNQ3, KCNQ4 and KCNQ5 subunits. Single-cell PCR of dissociated hippocampal pyramidal neurons gave detectable signals for only KCNQ2, KCNQ3 and KCNQ5; where tested, most also expressed mRNA for the vesicular glutamate transporter VGLUT1. Staining for KCNQ2 and KCNQ5 protein showed punctate fluorescence on both the somata and dendrites of hippocampal neurons. Staining for KCNQ3 was diffusely distributed whereas KCNQ4 was undetectable. In perforated patch recordings, linopirdine, a specific M-channel blocker, fully inhibited $I_{K(M)}$ with an IC_{50} of 3.6 ± 1.5 μM. In 70 % of these cells, TEA fully suppressed $I_{K(M)}$ with an IC_{50} of 0.7 ± 0.1 mM. In the remaining cells, TEA maximally reduced $I_{K(M)}$ by only 59.7 ± 5.2 % with an IC_{50} of 1.4 ± 0.3 mM; residual $I_{K(M)}$ was abolished by linopirdine. Our data suggest that KCNQ2, KCNQ3 and KCNQ5 subunits contribute to $I_{K(M)}$ in these neurons and that the variations in TEA sensitivity may reflect differential expression of KCNQ2, KCNQ3 and KCNQ5 subunits.

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Corresponding author D. A. Brown: Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, UK. Email: d.a.brown@ucl.ac.uk

M-type K^+ currents ($I_{K(M)}$) are non-inactivating voltage-dependent K^+ currents that play a key role in regulating neuronal firing frequency and excitability (Brown, 1988). They were originally described in sympathetic neurons but have subsequently been identified in a variety of neurons, including mammalian hippocampal pyramidal cells (Selyanko & Sim, 1998; see Brown, 1988, for antecedent reports). Selective inhibition of the M-current in hippocampal neurons with, for example, linopirdine reduces spike discharge accommodation and enhances repetitive firing (Aiken *et al.* 1995).

Recent evidence suggests that the M-channels in rat sympathetic neurons are composed of a heteromeric assembly of KCNQ2 and KCNQ3 gene products (Wang *et al.* 1998). Two other neuronal members of the KCNQ family, KCNQ4 and KCNQ5, have also been cloned (see Jentsch, 2000). Though the expression pattern of KCNQ4 is largely restricted to the central auditory system and the hair cells of the inner ear (Kharkovets *et al.* 2000), KCNQ2, 3 and 5 are widely distributed in the central nervous system, including the hippocampus (Wang *et al.* 1998; Cooper *et al.* 2000; Lerche *et al.* 2000; Schroeder *et al.* 2000; Saganich *et al.* 2001). When heterologously expressed, all members of the neuronally expressed KCNQ family can generate 'M-currents' as defined biophysically and pharmacologically (Selyanko *et al.* 2000; Lerche *et al.* 2000; Schroeder *et al.* 2000), and both KCNQ4 and KCNQ5, as well as KCNQ2, can form heteromultimers with KCNQ3 (see Jentsch, 2000). Hence, the subunit composition of native M-channels may show considerable diversity between different neurons.

In the present study, we have sought evidence regarding the molecular composition of M-channels in postnatal rat hippocampal neurons in culture from the *in situ* distribution of KCNQ channel subunits and from the pharmacology of native M-currents.

METHODS

Hippocampal cell culture

All experimentation was conducted according to the provisions of the UK Animals (Scientific Procedures) Act 1986. Rat pups (5–7 days old) were decapitated. Hippocampal CA1 and CA3 neurons were isolated as described by Shah & Haylett (2000), and maintained in culture using Neurobasal medium supplemented with 0.5 % (w/v) L-glutamine, 2 % B27 serum free supplement and

0.02 mg ml^{-1} gentamicin. Electrophysiological recordings were obtained from cells in culture for 8–15 days. Pyramidal cells were identified *ab initio* by their size and morphology, and subsequently by their electrophysiological characteristics, with complementary tests for expression of transmitter-related enzymes (see Results).

CHO cell culture

Chinese hamster ovary (CHO) cells were cultured and transfected as described by Hadley *et al.* (2000). Cells were grown in alpha-MEM medium supplemented with 10 % fetal calf serum (FCS), 1 % L-glutamine and 1 % penicillin–streptomycin in 95 % O_2–5 % CO_2. Cells were plated on glass coverslips and transfected with plasmids containing KCNQ4 cDNA using LipofectAmine Plus (Life Technologies), according to the manufacturer's instructions.

Reverse transcription-PCR (RT-PCR)

RT-PCR was performed on CA1 and CA3 regions in freshly dissected hippocampi from 5- to 7-day-old rats (i.e. at the age taken for subsequent cell culture and electrophysiology). DNase I-treated RNA (0.2 μg) was reverse transcribed using M-MLV reverse transcriptase (Promega). One-tenth of the resulting cDNA template was subjected to PCR amplification using oligodeoxynucleotide primers based on rat (KCNQ2, GenBank accession no. AF08453; KCNQ3, AF091247; KCNQ4, AF249748) or human (KCNQ5, AF202977) sequences. Primers were designed to be intron spanning (based on the human KCNQ genes) and were as follows:

rKCNQ2 (2900s): AGTGCGGATCAGAGTCTC;

rKCNQ2 (3126a): GCTCTGATGCTGACTTTGAGGC;

rKCNQ3 (746s): CAGCAAAGAACTCATCACCG;

rKCNQ3 (906a): ATGGTGGCCAGTGTGATCAG;

rKCNQ4 (40s): CCCTCCAAGCAGCATCTG;

rKCNQ4 (420a): TTGATTCGTCCCAGCATGTCCA;

hKCNQ5 (995s): GGAACCCAGCTGCCAACCTCAT;

hKCNQ5 (1101s): CTTTCTTGGTAGGGCTGCAG.

The cycling conditions used were: 1 cycle at 94 °C for 3 min; 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; 1 cycle at 72 °C for 5 min. A higher annealing temperature of 60 °C was used for KCNQ3 and KCNQ5 primers. Amplified products were analysed by electrophoresis through 2 % MetaPhor agarose (FMC BioProducts).

Single-cell PCR

The method used here was adapted from Zawar *et al.* (1999). Cytosol from single hippocampal pyramidal neurons (obtained from 5- to 7-day-old rats and cultured for 8–15 days as for the electrophysiological experiments) was collected into 7.5 μl of recording solution and eluted into a tube containing 2.5 μl first strand buffer (2 mM each deoxynucleotide triphosphate, 20 μM oligo d(T)$_{15}$, 40 mM dithiothreitol and 20 U RNase inhibitor (Roche)). Reverse transcription of mRNA transcripts was initiated by addition of 100 U M-MLV reverse transcriptase RNase H (−) point mutant (Promega) followed by incubation at 37 °C for 1 h. A multiplex PCR protocol was then used to amplify simultaneously cDNA for KCNQ2–5, and vesicular glutamate transporters VGLUT1 and VGLUT2. Primers for KCNQ2–5 were as described above. Primers for VGLUT1 and VGLUT2 were:

VGLUT1 (s): ATAATGTCCACGACCAATGTG;

VGLUT1 (a): GAGGCTATGAGGAACACGTAC;

VGLUT2 (s): GCTCCAAGATATGCCAGTATC;

VGLUT2 (a): GGTTGTTTCTCTCCTGAGGCA.

Multiplex PCR amplification was performed by addition of 5 U *Taq* polymerase (Promega) in a standard PCR buffer to 10 μl of single-cell RT product to give a final volume of 70 μl. The primers (10 pmol each) were added in a volume of 30 μl. PCR amplification consisted of 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 40 s, followed by elongation for 10 min at 72 °C. The products of the multiplex amplification were purified using the 'High Pure' PCR purification kit (Roche). Gene-specific PCRs were then performed on 3 μl of amplified cDNA template.

Immunofluorescence

Cells cultured on glass coverslips were fixed with 4 % paraformaldehyde for 20 min and permeabilized in phosphate-buffered saline (PBS) containing 0.1 % Triton X-100 for 15 min. A blocking solution of 10 mg ml^{-1} BSA in PBS was applied for 1 h, followed by overnight incubation in PBS containing 1 mg ml^{-1} BSA plus the primary antibody. The primary antibodies used were: rabbit anti-KCNQ2 (1:100; Santa Cruz Biotechnology), anti-KCNQ3 (1:100; Santa Cruz Biotechnology), anti-KCNQ4 (1:500; a gift from T. J. Jentsch) and anti-KCNQ5 (1:500; a gift from A. Villarroel); and goat polyclonal anti-GAD65 (glutamic acid decarboxylase, 1:100; N-19 from Santa Cruz Biotechnology). Following several washes, TRITC- or FITC-coupled secondary antibodies (1:100/1:200; DAKO Corporation, CA, USA) were applied for 1 h at room temperature. After several more washes cells were attached to microscope slides using a fluorescence mounting medium (DAKO Corporation) and visualized with a confocal microscope. Specificity of the KCNQ2, KCNQ3 and KCNQ4 antibodies was determined by competing out the staining by pre-absorbing the antibodies with the relevant immunogenic peptides (20:1 excess).

Perforated patch-clamp recording

Currents were recorded using the perforated patch-clamp method at 30 °C. Cells were superfused at a flow rate of 5 ml min^{-1} with a bathing solution of the following composition (mM): NaCl, 130; KCl, 3; $CaCl_2$, 2.5; Hepes free acid, 5; glucose, 10; $NaHCO_3$, 26; tetrodotoxin (TTX), 0.001; pH maintained at 7.2 with 95 % O_2–5 % CO_2. Glass pipettes (Clark Electromedical Instruments) were coated with Sylgard, fire-polished and had resistances of 4–10 MΩ when filled with an internal solution consisting of (mM): $KMeSO_4$, 126; KCl, 14; Hepes, 10; $MgCl_2$, 3; and containing 1.2 mg ml^{-1} amphotericin B (pH 7.25 with KOH). Cells were voltage clamped using the discontinuous voltage-clamp mode (sampling rate 3–5 kHz) of an Axoclamp 2A amplifier (Axon Instruments). M-currents were obtained by applying 1 s hyperpolarizing steps to –50 mV from a holding potential of –20 mV every 30 s and low-pass filtered at 3 kHz. Digitized signals were acquired on a computer using pCLAMP6 software (Axon Instruments). Drugs were applied by switching to a superfusion fluid containing the drug using a multi-way tap. The inlet tube was positioned such that the flow was directed onto the patched cell.

Data analysis

Data were analysed using pCLAMP6. $I_{K(M)}$ amplitude was typically measured from deactivation relaxations at –50 mV averaged from two successive records. Results are expressed as means ± S.E.M. Where appropriate, Student's *t* tests were used to determine statistical significance. The concentration–inhibition curves were fitted with the Hill equation:

$$y = y_{max}[I]^{n_H}/(K^{n_H} + [I]^{n_H}), \quad (1)$$

where y is the percentage inhibition, [I] is the drug concentration, y_{max} is the maximum inhibition, n_H is the Hill coefficient and K is the IC_{50} value.

Materials

All solutions and chemicals were obtained from Sigma except for Neurobasal medium, B27 serum free supplement, L-glutamine, penicillin–streptomycin and FCS, which were obtained from Life Technologies, UK. $KMeSO_4$ was purchased from Pfaltz and Bauer, Inc.

RESULTS

We first investigated which KCNQ subunits are expressed in the hippocampus when freshly dissected from 5- to 7-day-old rats by RT-PCR using intron-spanning primers. KCNQ2, KCNQ3, KCNQ5 and KCNQ4 mRNAs were detected in both CA1 and CA3 regions (Fig. 1*A*).

Single-cell PCR from hippocampal pyramidal neurons that had been in culture for 8–10 days subsequent to their isolation consistently (7 out of 7 tested) showed mRNA transcripts for KCNQ2, KCNQ3 and KCNQ5 subunits, but not for KCNQ4 (Fig. 1*B*). In four of these cells so tested, three showed an additional positive reaction for mRNA transcripts for the glutamate transporter VGLUT1 (Fig. 1*B*); one of them also gave a weak signal for VGLUT2 (data not shown). These results indicate that the neurons were glutamatergic (e.g. Fremeau *et al.* 2001). No signals were detected in the absence of M-MLV reverse transcriptase (data not shown).

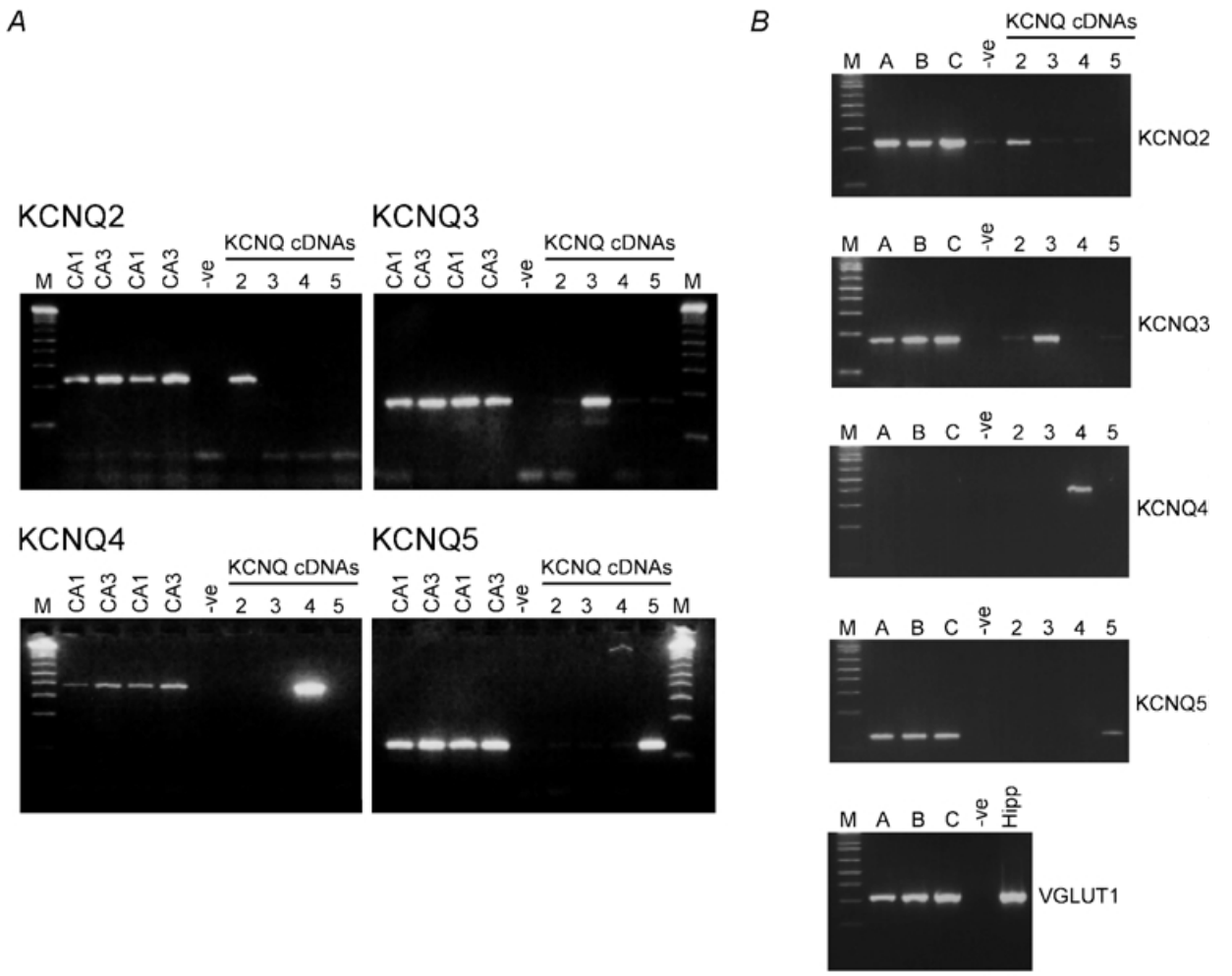

Figure 1. Reverse-transcription PCR analysis from rat pyramidal hippocampal neurons

A, RT-PCR was performed using 0.2 μg of DNase I-treated, total RNA from CA1 or CA3 hippocampal regions isolated from two different 5- to 7-day-old rats. See Methods for intron-spanning primer pairs. Control reactions were performed using plasmids containing cDNA sequences encoding rKCNQ2, rKCNQ3, rKCNQ4 and hKCNQ5 (labelled 2 to 5). −ve, absence of template; M, 1 kb plus ladder (Life Technologies). *B*, representative single-cell PCRs for KCNQ2–5 and VGLUT1 mRNAs obtained from three single hippocampal neurons (labelled A, B and C). Cells were isolated from the CA1 and CA3 regions of 7-day-old rats and subsequently cultured *in vitro* for 10 days. Primer pairs used are described in Methods. Control reactions were performed using plasmids containing cDNA sequences encoding rKCNQ2, rKCNQ3, rKCNQ4 and hKCNQ5 (labelled 2 to 5). Hipp, rat hippocampus cDNA; −ve, absence of template; M, 1 kb plus ladder.

Confocal immunofluorescence microscopy of the cultured cells revealed staining for KCNQ2 and KCNQ5 on the somata and dendrites of most (if not all) pyramidal neurons (Fig. 2). KCNQ2 somato-dendritic staining appeared to predominantly label the cell surface. KCNQ5 staining labelled both the cytosol and the cell surface, the latter being most prominent along processes. Hot spots of KCNQ5 staining were often seen on the neuropil of hippocampal neurons, perhaps indicating presynaptic structures. KCNQ3 staining was variable from cell to cell and clearly detectable in only ~30 % of pyramidal neurons; when detected (see Fig. 2), it was restricted to the cell body and large proximal dendrites, with staining of both cytoplasm and membrane. Staining for KCNQ4 was not detected, in agreement with the single-cell PCR data. We confirmed that our polyclonal anti-KCNQ4 antibodies recognize KCNQ4 proteins expressed in CHO cells (inset in Fig. 2). Large cells of this type showed no staining for GAD65; instead, GAD65 staining was restricted to small, round cells (data not shown) (see Cao *et al.* 1996).

We next characterized $I_{K(M)}$ pharmacologically in cultured neurons using perforated patch-clamp recordings. Recorded neurons showed the slow after-hyperpolarization (sAHP) currents characteristic of pyramidal cells (see Shah & Haylett, 2000, and references therein). When held at –20 mV, neurons displayed a steady outward current with an average magnitude of 540 ± 50 pA (n = 30). Application of the conventional M-current deactivation protocol (Brown & Adams, 1980) resulted in characteristic voltage- and time-dependent inward relaxations on stepping to –50 mV (Fig. 3*A* and *B*). Inward deactivation relaxations displayed biphasic kinetics with fast (23.3 ± 2.5 ms) and slow (680 ± 138 ms) time constants. As previously observed (Selyanko & Sim, 1998), stimulation of muscarinic receptors

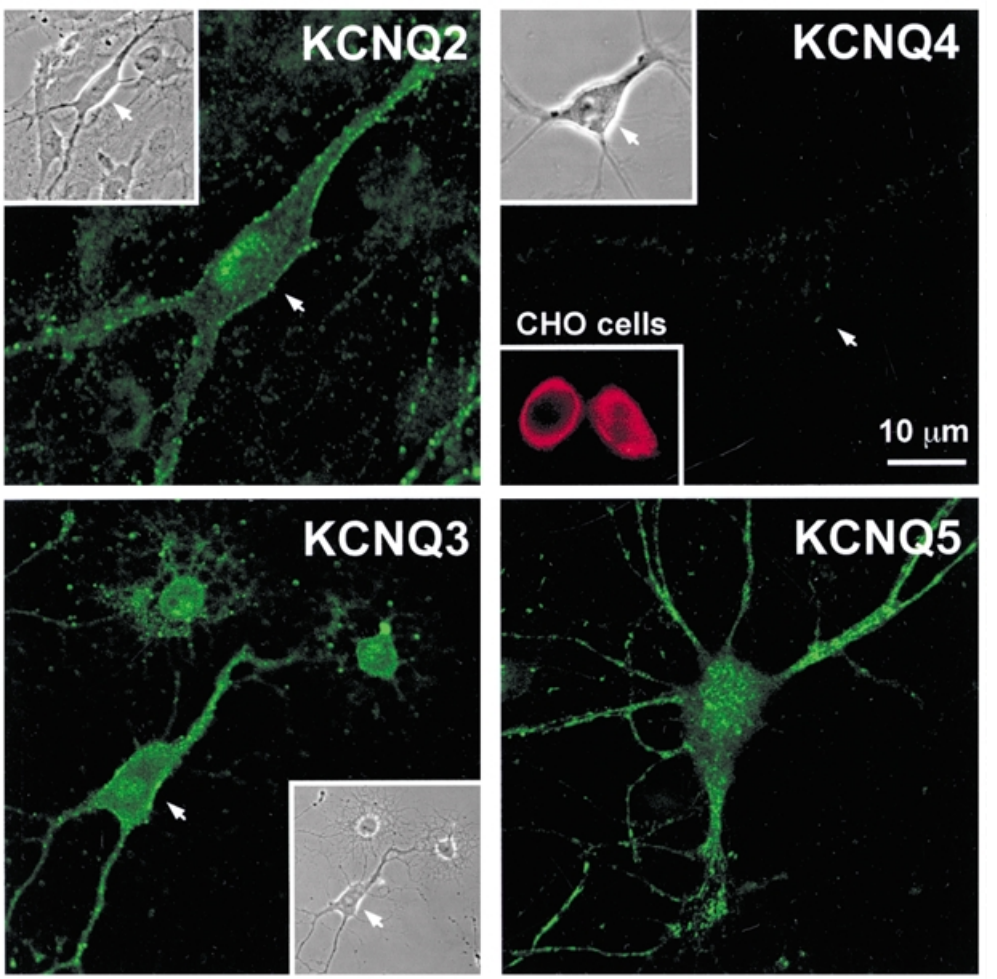

Figure 2. Immunodetection of KCNQ channel subunits in hippocampal neurons

Confocal images (10 stacked at 0.5 μm intervals) of KCNQ2, KCNQ3, KCNQ4 and KCNQ5 immunostaining in hippocampal pyramidal neurons cultured *in vitro* for 10 days. Note the plasma membrane staining for KCNQ2 whereas KCNQ3 and KCNQ5 antibodies appear to label both the cell surface and intracellular components. Inset, staining of CHO cells expressing exogenous KCNQ4 subunits with the anti-KCNQ4 antibody. Scale bar, 10 μm. The greyscale insets show phase-contrast images of the same fields at 40 % magnification.

by the muscarinic agonist oxotremorine-M (10 μM) reduced the outward current and strongly inhibited $I_{K(M)}$ deactivation relaxations ($n = 5$), the difference current showing a characteristic $I_{K(M)}$ trajectory (Fig. 3*A*).

Linopirdine, a specific blocker of native $I_{K(M)}$ (Aiken *et al.* 1995; Schnee & Brown, 1998) and of expressed KCNQ channels (Wang *et al.* 1998), also dose-dependently reduced $I_{K(M)}$ inward relaxations (Fig. 3*B*). Block was total with an IC_{50} value of 3.6 ± 1.5 μM (Fig. 3*C*).

To test for a differential contribution of KCNQ2 and KCNQ5 subunits we took advantage of their different sensitivities to TEA: this inhibits KCNQ2 at well-under millimolar concentrations (Wang *et al.* 1998; Hadley *et al.* 2000), whereas it has very little effect on KCNQ5 (Lerche *et al.* 2000; Schroeder *et al.* 2000). In 70 % (7/10) of cells tested, TEA (10 mM) fully inhibited (by 96.1 ± 6.2 %) $I_{K(M)}$ relaxations (Fig. 4*A*) with an IC_{50} of 0.7 ± 0.1 mM and a mean 'Hill slope' of 0.77 ($n = 7$; Fig. 4*C*). Subsequent application of linopirdine (30 μM in the presence of 10 mM

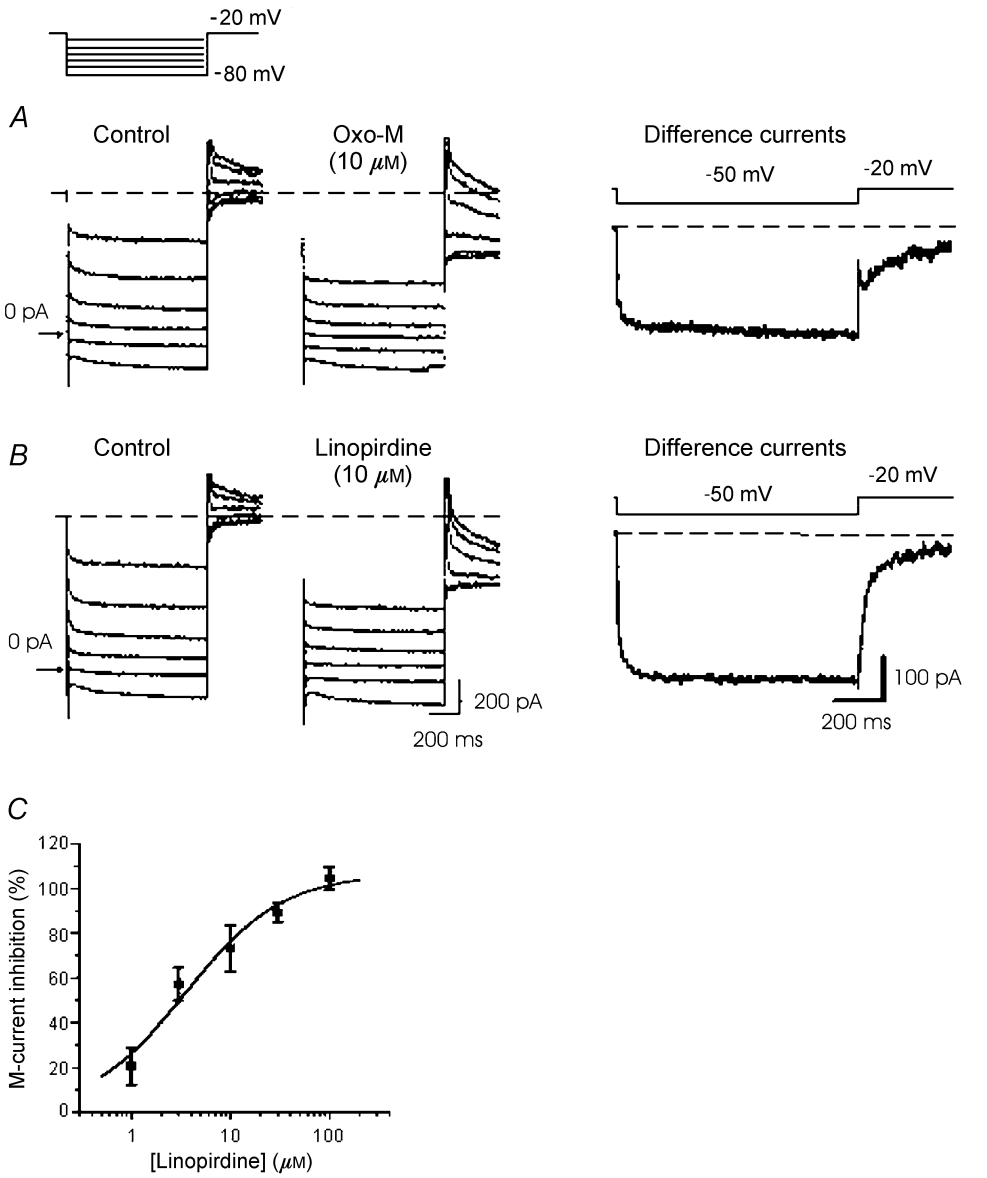

Figure 3. Pharmacological characterization of $I_{K(M)}$ in hippocampal pyramidal cells

A and *B* show currents recorded from two cultured neurons in the absence (Control) and presence of oxotremorine-M (Oxo-M) and linopirdine, respectively. Cells were held at –20 mV and currents evoked by stepping to –50 mV (voltage protocol shown in *A*). The difference currents were obtained by subtracting the control trace at −50 mV from that in the presence of the respective compounds. The horizontal dashed lines mark the initial baseline holding current. The scale bars shown in *B* also apply to *A*. *C*, average concentration–inhibition curve for linopirdine fitted using eqn (1). Each data point is the mean ± S.E.M. of 3–13 observations. (The slow component of the difference current in *A* may reflect the hyperpolarization-activated cation current I_Q/I_h, which is enhanced by oxotremorine-M: see Colino & Halliwell, 1993.)

TEA) produced no further block ($n = 7$; Fig. 4*A*), indicating that the effects of linopirdine and TEA were occlusive.

In 30 % (3/10) of cells tested, however, TEA only partially inhibited $I_{K(M)}$ inward relaxations (by 59.7 ± 5.2 %, Fig. 4*B*). Nevertheless, the IC_{50} value (1.4 ± 0.3 mM) for block by TEA did not differ significantly from that of cells exhibiting full inhibition of $I_{K(M)}$ by TEA (Fig. 4*C*). Further application of linopirdine (30 μM) in the presence of TEA (10 mM) abolished the residual $I_{K(M)}$ (Fig. 4*B*). This indicates that the residual current was carried by KCNQ channels and that incomplete inhibition by TEA was not due to clamp-escape. (Although, at 30 μM, linopirdine might produce some inhibition of other K^+ channel currents such as Kv1.2 and Kv4.3 (Wang *et al.* 1998), these would not contribute to the standing current recorded at –20 mV.)

DISCUSSION

The present experiments represent the first attempt at identifying the KCNQ channel subunits responsible for the M-current in hippocampal neurons. We have used hippocampi dissected from 5- to 7-day-old rats, assessed the expression of different KCNQ mRNAs in hippocampal tissue at that stage by RT-PCR and then assessed the expression of mRNAs and protein, and their contributions to recorded currents, in individual pyramidal neurons after subsequent culture for 8–15 days. We recognize that this does not necessarily represent the situation at other stages of development, nor in adult neurons *in situ*. Nevertheless, cultured neurons are frequently used for electrophysiological and other studies, and previous work on the expression of members of the Kv channel family

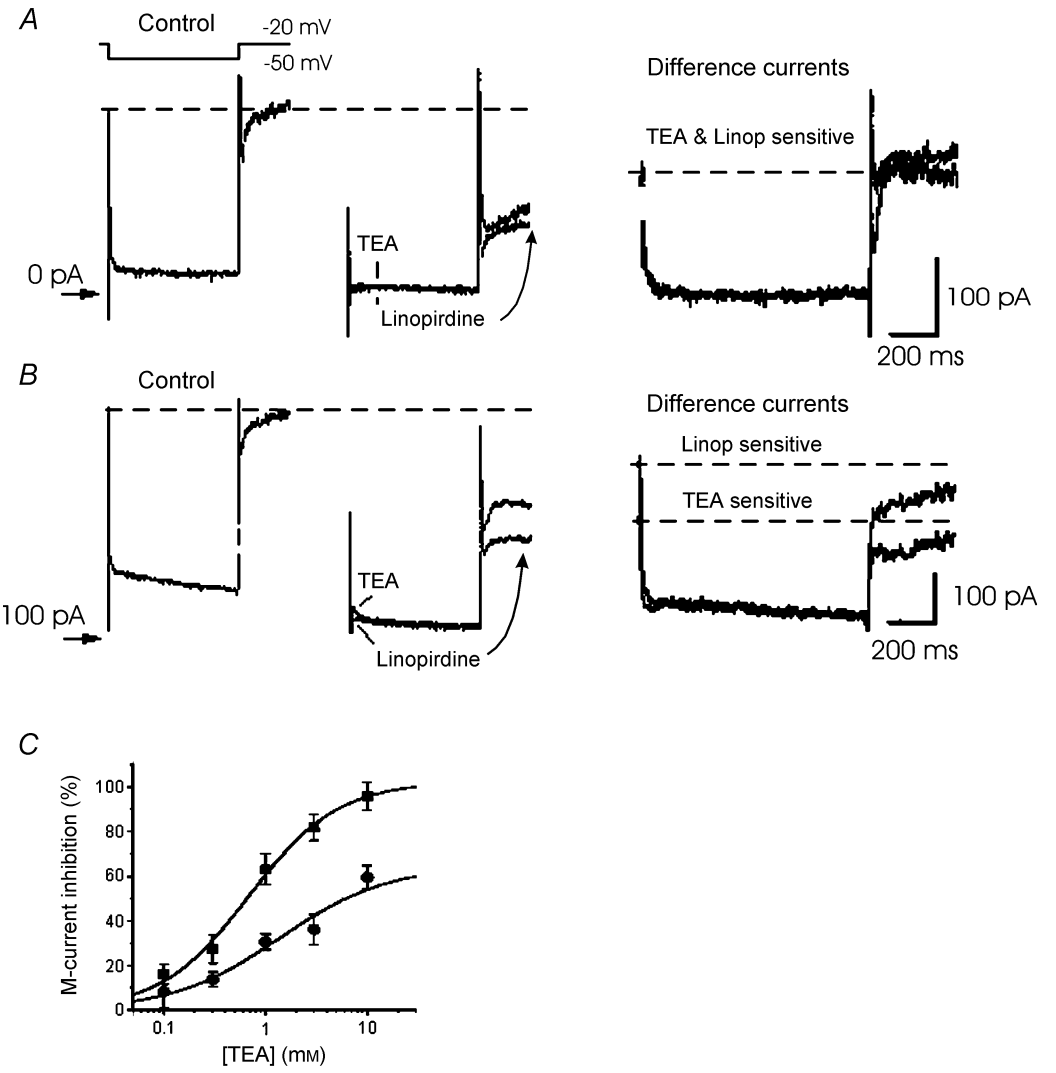

Figure 4. Effects of TEA on $I_{K(M)}$ recorded in hippocampal pyramidal neurons

A and *B* show representative examples of currents recorded from two different cells with differential sensitivity to 10 mM TEA. Superimposed are currents in the presence of TEA and co-applied TEA and linopirdine (Linop, 30 μM). The currents were recorded by applying the voltage step shown in *A*. *C*, cumulative concentration–inhibition curves for TEA. Squares and circles represent curves for cells in which TEA abolished $I_{K(M)}$ ($n = 7$) and cells in which TEA only partially inhibited $I_{K(M)}$ ($n = 3$), respectively.

suggests that, while the precise topographical distribution of Kv K^+ channel subunits in cultured cells may not fully reproduce that obtaining *in situ*, their overall developmental expression *in vitro* provides a fair qualitative match to that *in situ* (Maletic-Savatic *et al.* 1998; Grosse *et al.* 2000).

We consider the cells studied to have been pyramidal cells on the basis of: (1) morphology; (2) the presence of a long-lasting (> 1 s) after-hyperpolarization current following a depolarizing prepulse (Shah & Haylett, 2000), characteristic of pyramidal cells (Lancaster & Adams, 1986; Storm, 1990), but not of interneurons (Savic *et al.* 2001); (3) the presence of mRNA for the vesicular glutamate transporter VGLUT1 in single-cell PCR assays; and (4) absence of immunofluorescence staining for GAD65 protein. (GAD65 mRNA has been detected in hippocampal pyramidal cells by single-cell PCR and *in situ* hybridization, though not necessarily accompanied by immunodectable protein (Cao *et al.* 1996).)

While all four transcripts (KCNQ2, 3, 4 and 5) could be detected in CA1/CA3 regions of the entire hippocampus dissected from 5- to 7-day-old rats by RT-PCR, single-cell PCR of subsequently cultured neurons suggests that KCNQ2, KCNQ3 and KCNQ5 gene products were most abundantly expressed in the pyramidal cells. Immunocytochemistry provided concordant evidence for expression of KCNQ2 and KCNQ5 protein, but clear staining for KCNQ3 protein was only apparent in a minority of cells. Since high levels of KCNQ3 mRNA have been reported in adult rat hippocampi (Wang *et al.* 1998) and in adult CA1 and CA3 pyramidal cells by *in situ* hybridization (Saganich *et al.* 2001), this may reflect the late developmental expression of KCNQ3 transcripts (as previously noted in mice; Tinel *et al.* 1998), and consequent low protein levels. The absence of detectable KCNQ4 mRNA and protein in single hippocampal neurons accords with its primary expression in the auditory and vestibular pathway (Kharkovets *et al.* 2000). On the other hand, detection of KCNQ4 signals in hippocampal tissue by RT-PCR accords with previous results with mouse whole brain tissue (Kubisch *et al.* 1999) and may reflect low levels of mRNA expression in other hippocampal neurons, fibres or glial cells.

In electrophysiological recordings, a sustained outward current yielding biphasic M-like relaxations during hyperpolarizing steps was identified. In agreement with previous observations (see Introduction), these relaxations were inhibited by the muscarinic agonist oxotremorine-M. They were also completely inhibited by linopirdine, with an IC_{50} of 3.6 μM – in reasonable agreement with previous measurements in adult hippocampal slices (8.5 μM: Aiken *et al.* 1995; 2.4 μM: Schnee & Brown, 1998) and on expressed KCNQ2–3 (4 μM: Wang *et al.* 1998) and KCNQ3–5 currents (7.7 μM: Wickenden *et al.* 2001).

Because of the variable presence of a tyrosine, threonine or valine in the upper pore region, TEA can discriminate between different KCNQ subunits (Wang *et al.* 1998; Hadley *et al.* 2000). It has previously been reported that M-currents in adult rat hippocampal slices are substantially reduced by 5 mM TEA (Storm, 1989). In the present experiments, the M-current relaxations were completely inhibited by TEA in 70 % of cells tested, with an IC_{50} of 0.7 mM. This is less than the IC_{50} for block of expressed heteromeric KCNQ2–3 currents (3.7 mM: Wang *et al.* 1998; 3.8 mM: Hadley *et al.* 2000), though somewhat higher than the IC_{50} for block of homomeric KCNQ2 currents (0.16 mM: Wang *et al.* 1998; 0.3 mM: Hadley *et al.* 2000; 0.17 mM: Shapiro *et al.* 2000). This suggests that, in these cells, KCNQ2 subunits provide a dominant contribution to the channels carrying the native M-current – possibly as a mixture of homomeric KCNQ2 channels and heteromeric KCNQ2–3 channels. This would accord with the results of the immunofluorescence studies showing relatively low and variable levels of KCNQ3 protein. Channel subunit heterogeneity is also supported by the rather shallow slope (0.77) of the concentration–inhibition curve (see Shapiro *et al.* 2000).

On the other hand, in 30 % of neurons tested, TEA only blocked around 60 % of the M-current at a concentration of 10 mM. The blocked component of current was not significantly less sensitive to TEA than that in those cells in which 10 mM TEA produced full block, so it might similarly reflect current through KCNQ2 and KCNQ2–3 channels. However, since the residual current was completely suppressed by linopirdine, this must be carried by other KCNQ channels appreciably less sensitive to TEA. From the single-cell PCR and immunohistochemical data, the most plausible origin of this component of current is homomeric KCNQ5 and/or heteromeric KCNQ5–3 channels, since both are insensitive to TEA at < 10 mM (Lerche *et al.* 2000; Schroeder *et al.* 2000). However, we observed a discrepancy between electrophysiological and immunological data in that most pyramidal neurons were stained for KCNQ5 but only ~30 % of them displayed an M-current component resistant to TEA. This may be explained by the fact that membrane staining for KCNQ5 was most strongly associated with neuron processes, rather than the somatic membrane (Fig. 2), suggesting that KCNQ5 might contribute primarily to dendritic $I_{K(M)}$ and to a lesser (and variable) extent to somatic $I_{K(M)}$.

We think it less likely that homomeric KCNQ3 channels contribute significantly to the TEA-resistant component of M-current, for two reasons. First, KCNQ3 transcripts generated substantially smaller currents than KCNQ5 transcripts when expressed in oocytes (Lerche *et al.* 2000; Schroeder *et al.* 2000). Second, KCNQ3 subunits traffic much more effectively to the surface membrane in heteromeric form than in homomeric form (Schwake *et al.*

2000). Since KCNQ3 subunits can form heteromeric channels with both KCNQ2 and KCNQ5, whereas KCNQ2 and KCNQ5 subunits do not appear to co-assemble with each other (Jentsch, 2000), a substantial fraction of homomeric KCNQ3 channels would only be expected if there were a large excess of KCNQ3 transcripts over KCNQ2 and KCNQ5 transcripts; our PCR and immunocytochemical data suggest this to be unlikely.

In conclusion, our data indicate that KCNQ2 and KCNQ5 gene products can contribute to native somatic M-currents in these cultured rat hippocampal cells, partly as heteromultimers with KCNQ3 but also probably as homomultimers. This latter feature may be a consequence of the fact that KCNQ3 protein appeared to be less strongly expressed. Perhaps, as in mice (Tinel *et al.* 1998), KCNQ3 expression increases during development, leading to a progressive switch towards heteromeric KCNQ2–3 and KCNQ3–5 channels in adulthood. Possibly KCNQ5 subunits may also make a stronger contribution to currents in dendritic or axonal processes. Nevertheless, the present results provide the first intimation of a contribution by KCNQ5 subunits to native neuronal currents.

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Authors' present addresses

M. M. Shah: Division of Neuroscience, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA.

P. Delmas: Intégration des Informations Sensorielles (ITIS)CNRS, 31 Chemin Joseph Aiguier, 13402 Marseille cedex 20, France.