KCNQ/M-currents contribute to the resting membrane potential in rat visceral sensory neurons

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The M-current is a slowly activating, non-inactivating potassium current that has been shown to be present in numerous cell types. In this study, KCNQ2, Q3 and Q5, the molecular correlates of M-current in neurons, were identified in the visceral sensory neurons of the nodose ganglia from rats through immunocytochemical studies. All neurons showed expression of each of the three proteins. In voltage clamp studies, the cognition-enhancing drug linopirdine (1–50 \( \mu \text{M} \)) and its analogue, XE991 (10 \( \mu \text{M} \)), quickly and irreversibly blocked a small, slowly activating current that had kinetic properties similar to KCNQ/M-currents. This current activated between −60 and −55 mV, had a voltage-dependent activation time constant of 208 ± 12 ms at −20 mV, a deactivation time constant of 165 ± 24 ms at −50 mV and \( V_{1/2} \) of −24 ± 2 mV, values which are consistent with previous reports for endogenous M-currents. In current clamp studies, these drugs also led to a depolarization of the resting membrane potential at values as negative as −60 mV. Flupirtine (10–20 \( \mu \text{M} \)), an M-current activator, caused a 3–14 mV leftward shift in the current–voltage relationship and also led to a hyperpolarization of resting membrane potential. These data indicate that the M-current is present in nodose neurons, is activated at resting membrane potential and that it is physiologically important in regulating excitability by maintaining cells at negative voltages.

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The visceral sensory neurons are critical in relaying information from peripheral sensory receptors to the nucleus of the solitary tract (NTS) in the brainstem. It is there that visceral sensory information from cardiovascular, respiratory and gastrointestinal afferents is integrated to generate the neural outflow that is essential for maintaining appropriate blood pressure, heart rate, respiration and gastrointestinal function. The afferent pathways that relay this information have particular characteristics that are unique for this purpose, including an axonal pathway that provides a direct transmission from the peripheral sensory receptor to the brainstem with a single side branch to the cell soma.

Under physiological conditions, the soma of the sensory neurons are generally thought not to contribute information to the activity arising at their peripheral receptor terminals, but simply to monitor the activity as it travels to the central terminations of the axons in the NTS. It is essential then that the soma maintain a stable, negative resting membrane potential (−50 to −60 mV) to ensure that they fire only when sensory information from the peripheral receptor terminal is received. The underlying basis of this stability is not completely known. At the resting membrane potential, the hyperpolarization-activated, cyclic-nucleotide-gated (HCN) family of ion channels is active, driving the membrane potential toward the reversal of about −35 mV. This depolarizing drive is offset by undetermined potassium current(s) (Doan & Kunze, 1999). In this study we sought to identify the potassium current contributing to the resting membrane potential. A potential candidate which had not previously been identified in these cells was the M-current (\( I_M \)). The M-current is a slowly activating, non-inactivating, voltage-dependent potassium (\( K^+ \)) current that activates in the range of the resting membrane potential. It acts to maintain the membrane potential below the threshold for sodium current activation, thereby regulating neuronal firing and excitability (Marrion, 1997). Recent work has suggested that the M-current is carried by three members of the KCNQ family of ion channel proteins, KCNQ2, Q3 and Q5 (Wang et al. 1998; Lerche et al. 2000; Schroeder et al. 2000; Wickenden et al. 2001; Shah et al. 2002; Roche et al. 2002). These subunits have a relatively restricted distribution in that they are primarily confined
to the nervous system. The importance of these channels in maintaining cells at resting potential is illustrated by the observation that mutations in KCNQ2 and KCNQ3 play a role in causing benign familial neonatal convulsions (BFNC), a form of epilepsy (Jentsch, 2000).

In the current study we have identified the presence of KCNQ2, Q3 and Q5 in rat nodose ganglion cells. Using the known M-current blockers, linopirdine and XE991, we isolated an M-like current from other endogenous potassium currents. The current was slowly activating and non-inactivating with properties similar to those of M-current and its suppression by the blockers was irreversible over 10 min. In current clamp studies, application of the blocker XE991 caused a depolarization of resting membrane potential. To further demonstrate the presence of M-current, we showed that application of the M-current enhancer, flupirtine, increased current in these cells, led to a hyperpolarization of the resting membrane potential, and caused a leftward shift in the current–voltage relationship that is characteristic of M-current in the presence of this drug.

**Methods**

**Isolation and culture of nodose ganglia**

Neonatal (postnatal days 0–2) Sprague-Dawley rats were asphyxiated by CO₂ inhalation and the nodose ganglia were extracted in accordance with the Case Western Reserve University Animal Research Committee guidelines. The ganglia were collected in cold Nodose Complete Media (NCM: DMEM-F12, (Gibco) supplemented with 5% fetal bovine serum (HyClone) and 1% penicillin–streptomycin–neomycin antibiotic mixture (Gibco)) then incubated for 30 min at 37°C in Earle’s Balanced Salt Solution (ICN) containing 1 mg ml⁻¹ collagenase type 2 (Worthington). The enzyme solution was then replaced by 37°C NCM containing 1.5 mg ml⁻¹ albumin (bovine; Sigma) and the tissue was triturated with a fire-polished pipette to dissociate the cells. The cell suspension was then plated into 35 mm Petri dishes containing poly d-lysine-coated glass coverslips. The cells were used within 48 h of plating.

**Immunocytochemistry**

Neonatal nodose ganglia were harvested and plated as described above. After 4 h in culture, the cells were rinsed twice for 5 min in PBS then fixed in 4% paraformaldehyde in 0.1 m phosphate buffer for 15 min. After another short rinse in PBS, the cells were permeabilized in a 0.01% saponin solution for 10 min. Following permeabilization, the cells were blocked for 30 min in PBS containing 1% bovine serum albumin (BSA; w/v; Jackson) and 10% normal donkey serum (NDS; v/v; Jackson). Commercial primary antibodies used were guinea pig anti-PGP at 1:500 (Antibodies Inc.), rabbit anti-KCNQ2 at 1:200 (Chemicon), rabbit anti-KCNQ3 at 1:2000 (Chemicon) and rabbit anti-KCNQ5 at 1:500 (Chemicon). Antibody solutions were made in PBS with 1% BSA and incubated overnight at 4°C. One coverslip of cells was incubated with only PBS containing 1% BSA to serve as a negative control. Cells were washed four times for 10 min each in PBS then incubated in secondary antibody solution containing PBS, 1% BSA, 10% NDS, donkey anti-rabbit RedX at 1:300 (Jackson), and donkey anti-guinea pig FITC at 1:500 (Jackson) for 1.5 h at room temperature in the dark. Cells were washed four times for 10 min in PBS then coveredlipped with vextashield containing DAPI (Vector). Images were obtained using a Nikon Eclipse E600 microscope and a SPOT camera with SPOT advanced software (Diagnostic Instruments, Inc.).

**Electrophysiology**

Electrophysiological experiments were performed on nodose neurons at room temperature or 35°C, 24–48 h after plating. Using a whole-cell patch configuration under voltage- or current-clamp conditions, data were obtained with an Axopatch-1C patch clamp apparatus then digitized and analysed using pCLAMP programs (Axon Instruments) and Origin 7.5 (OriginLabs). Electrodes (2–4 MΩ) were prepared from 8161 glass (Garner Glass). The extracellular solution for voltage-clamp experiments contained (mM): 140 N-methyl-d-glucamine, 5.4 KCl (2.5 KCl was used for deactivation experiments only), 1 MgCl₂, 0.2 CaCl₂, and 10 Hepes, pH adjusted to 7.3 with HCl. 4-Aminopyridine (4-AP; 5 mM) was added to this bath solution in all voltage-clamp experiments to block numerous K⁺ currents not pertinent to our studies. N-methyl-d-glucamine does not exhibit any permeability through M channels (Block & Jones, 1996).
The extracellular solution for current-clamp studies contained (mm): 137 NaCl, 5.4 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and 10 Hepes, pH adjusted to 7.3 by NaOH. The pipette solution for voltage- and current-clamp contained (mm): 145 potassium aspartate, 2.2 EGTA, 10 Hepes, and 1 MgCl₂, pH adjusted to 7.2 by KOH. Stock solutions of linopirdine (Sigma), XE991 (Tocris) and flupirtine (Sigma) were made in ethanol, distilled water and dimethyl sulfoxide, respectively, and were stored at −20°C until they were further diluted in bath solution and perfused onto cells in voltage- and current-clamp experiments. The final concentration of the ethanol or DMSO was less than 0.2%. All values are mean ± s.e.m. unless otherwise noted. Maximum series resistance errors were estimated to be < 4 mV for M-current recordings (range of series resistance was 4.4–11.7 MΩ and was not compensated). HEK cells which stably expressed Kv2.1 (Kirsch et al. 1991) were provided by A. Brown (MetroHealth Medical Centre).

Results

Immunocytochemical analysis of isolated neonatal nodose ganglia cells in culture revealed the presence of KCNQ2, Q3 and Q5 (Fig. 1, top row; left, centre and right columns, respectively). The neuronal marker PGP was used to identify neurons in the culture (Fig. 1, second row) and distinguish them from other surrounding support cells. Co-localization of KCNQ and PGP labelling (Fig. 1, third row) showed all three of the KCNQ proteins tested were present in all neurons in the culture. We also used a second set of antibodies targeted against KCNQ2, Q3 and Q5 (raised in goat, Santa Cruz Biotechnologies) on cultured cells that produced the same staining patterns as those in Fig. 1, supporting the observation that all three proteins are present in nodose cells under these conditions and at this developmental stage.

We also performed immunohistochemistry for KCNQ2, Q3 and Q5 on sections of whole nodose ganglia (Fig. 1,
Separation of M-current from other endogenous potassium currents

We used patch-clamp techniques to isolate the M-current which is generally a small component of total outward potassium current (Brown & Yu, 2000). Since M-current is poorly blocked by 4-AP (Robbins, 2001), we added this drug at 5 mM to all of our voltage-clamp solutions to block the majority of the other potassium currents present in nodose neurons, and facilitate isolation of the KCNQ current. The currents present in nodose neurons blocked by 4-AP included Kv1.1, Kv1.2, Kv1.6 (Glazebrook et al. 2002) and Kv1.3, Kv1.5, Kv3.4 (unpublished data). We then used the M-current blockers, linopirdine, and its analogue XE991, to isolate the KCNQ current from the remaining 4-AP-insensitive current (Aiken et al. 1995; Lamas et al. 1997; Costa & Brown, 1997; Schnee & Brown, 1998). However, while these drugs are more selective for M-current, they have been shown to affect other currents (Schnee & Brown, 1998; Wang et al. 1998) and we found they affected two other currents in nodose neurons, in addition to M-current, that were still present due to their low sensitivity to 4-AP.

The first additional current blocked was transient (a Kv4.3-like current) and present in a subpopulation of the neonatal neurons at P0 and in most of the neurons by P3. Although the block by XE991 and linopirdine was weak, as previously reported (Wang et al. 1998), it interfered with M-current at the beginning of depolarizing steps positive to −20 mV, its activation threshold, making analysis of M-current activation over a range of voltages difficult. For that reason we evaluated the activation of the M-like current at −20 mV and/or, over a wider range, primarily in P1 cells where the transient current was either absent or only minimally developed.

The second additional current blocked by linopirdine and XE991 was a sustained current, like M-current, but was one that activated much faster. This 4-AP-insensitive current was present in a subpopulation of neurons and, in those neurons, presented a problem in studying M-current because, at potentials positive to −30 mV, it was active over the entire time course that M-current was activated. However, we were able to eliminate this current in the subpopulation where it was expressed because its block was rapidly reversible (within 2 s of washing), while block of the M-current was not reversible over the time course of the experiment (a similar irreversibility of M-current over this time course was also noted in previous studies by Schnee & Brown, 1998; Wickenden et al. 2000, 2001; Yue & Yaari, 2004; Yeung & Greenwood, 2005). Thus, we obtained the irreversibly blocked component representing the M-current by subtracting the current recorded during the wash period from the control current prior to administration of the drug. An example of this current isolation method is shown in the top half of Fig. 2. Currents were recorded from nodose neurons in control solution (Fig. 2A) and in the presence of 50 μM linopirdine (Fig. 2B). Upon washing out the drug with control solution (Fig. 2C), only part of the current block was reversible. Figure 2D was obtained by subtracting B from A and represents the total blocked current. By subtracting C from A, we obtained the irreversibly blocked current in Fig. 2E. Finally, the reversibly blocked current, Fig. 2F, is the subtraction of B from C. The total current blocked in Fig. 2D had both a fast component as well as a slower M-like current that increased over the entire pulse. The slower current was more readily apparent as the irreversibly blocked current (Fig. 2E), while reversibly blocked component is noticeably faster (Fig. 2F). The reversibly blocked current had activation time similar to Kv2.1 (τ = 20–40 ms at 0 mV). Using HEK cells induced to stably express Kv2.1 we confirmed the block of this channel by XE991, and more so by linopirdine, in a readily reversible manner (bottom half of Fig. 2). The series of four recordings in Fig. 2G represents currents in control solution, in the presence of 10 μM linopirdine, during a washout with control solution, and finally the blocked Kv2.1 current obtained by subtracting the second panel (+ Linopirdine) from the third panel (Wash). The series in Fig. 2H is the same as in Fig. 2G except 10 μM XE991 was used instead of linopirdine. We fitted the time course of the subtracted currents with a standard mono-exponential function with a tau of activation in the presence of linopirdine of 29 ± 4 ms (n = 8) and a tau in the presence of XE991 of 31 ± 5 ms (n = 8). Figure 2I illustrates suppression of Kv2.1 by varying concentrations of XE991 and linopirdine, as compared with a representative control recording. Figure 2J is the quantification of these data as normalized current. In Fig. 2I and J it can be seen that 10 μM XE991 suppressed the current by 6.5 ± 0.6% (n = 12), while 10 μM linopirdine suppressed the current by 7.0 ± 1% (n = 6). At 50 μM linopirdine, the current was blocked by 17.8 ± 2.5% (n = 6). At the high concentration of 100 μM, XE991 blocked Kv2.1 by 20.4 ± 0.8% (n = 5) and linopirdine suppressed the current by 34.2 ± 4.5% (n = 4).
Figure 2. Effects of linopirdine and XE991 on 4-AP-insensitive currents in nodose neurons and on Kv2.1 currents in HEK cells

Top half: currents were evoked from nodose neurons in the presence of 4-AP by the protocol in the upper right corner of the figure (800 ms pulses to voltages between −80 mV to +40 mV in 10 mV increments from a holding potential of −80 mV, followed by a step to −30 mV for 150 ms). A illustrates the 4-AP-insensitive currents in control solution. These currents decreased in the presence of 50 μM linopirdine (B) and partially recovered upon washout of the drug (C). The total current blocked in D was obtained by subtracting B from A, while the irreversibly blocked current (E) resulted from the subtraction of C from A. The component reversibly blocked by linopirdine (F)
Figure 3. Effects of XE991 on activation of M-current in nodose neurons
Experimental data illustrating current recorded in nodose neurons elicited by the protocol shown in the inset (an 800 ms pulse from a holding potential of −80 mV to a step voltage between −80 and +10 mV in 10 mV increments and finally back to −40 mV for 150 ms) in the absence (A) and presence (B) of 10 μM XE991 followed by a drug wash-out with bath solution (C). The irreversible XE991-sensitive current (D) was obtained by subtracting C from A to eliminate any reversible currents blocked by the drug which have been attributed to reversible block of Kv2.1. Activation time constants were determined from mono-exponential fits of the incremental pulses from −80 mV to the step voltages. The first 50 ms of the pulse were ignored during fitting due to the delay in M-current activation. The inset above D shows the mono-exponential fit (black line) overlaid on the original traces (grey) for steps to −20, 0 and 10 mV. E illustrates the activation curve of the data such as that in D, obtained from the tail current measurements (n = 15).

Pharmacological study of activation and deactivation of M-like current
When isolating M-current, we preferentially used XE991 over linopirdine because XE991 is more potent on M-current (Wang et al. 1998) and less potent on Kv2.1 (Fig. 2I and J). IC50 values for XE991 on M-currents are reported in the range of 1 μM (Wang et al. 1998). Since M-current in nodose is a small current we used 10 μM XE991 (as did Wang et al. 1998; Shah et al. 2002; Martire et al. 2004; Gu et al. 2005) to ensure that we blocked a majority of current to facilitate analysis. Also, this concentration was low enough that it blocked less than 7% of the Kv2.1 present (see Fig. 2 for concentration–response data).

Figure 3 shows a representative example of a cell without Kv2.1-like current, as illustrated by the persistent XE991 block even after returning to control solution (Fig. 3C). Figure 3A shows a substantial slowly activating current that was recorded during perfusion with bath solution and evoked by a long depolarizing pulse in 10 mV incremental steps from −80 to +10 mV, followed by a step back to −40 mV (see diagram in Fig. 3). XE991 (10 μM) reduced...
the current by 35% rapidly within 1 min of application (Fig. 3B). The current then continued to decrease slowly as more channels were bound by XE991. For this reason, upon washing (Fig. 3C), the current continued to decrease briefly before control solution removed the remaining XE991 from the bath. The slow time course of XE991 block has been previously reported (Martire et al. 2004; Gu et al. 2005; Peters et al. 2005; Yeung & Greenwood, 2005). We were not, in all cases, able to maintain a recording for the time it took to achieve steady-state block (approximately 10 min) and still complete the subsequent protocols. Therefore, we chose to examine the current after the initial rapid blocking phase. The subtracted current in Fig. 3D was obtained by subtracting Fig. 3C from Fig. 3A. This current has the characteristic shape of M-current with slow activation and lack of inactivation over the long pulse. Activation for this current consisted of two parts: an initial delayed rising phase (approximately 50 ms) followed by a slower activation phase (Main et al. 2000 and see Fig. 3 legend). By ignoring the initial rising phase, we were able to fit the slow activation with a single exponential function, as has been done for other potassium currents exhibiting an early delayed rising phase, such as Kv2.1 (Klemic et al. 1998). The current had a voltage-dependent activation time, with a value of 208 ± 12 ms at −20 mV (n = 11) and 102 ± 11 ms at +10 mV (n = 13). We were able to fit these currents without interference from the 4-AP-insensitive transient current discussed earlier because we limited our studies to primarily age P1 cells, and in a few P2 neurons, where the transient was minimally developed. The activation curve (Fig. 3E) was compiled from data of 15 cells and was plotted using the amplitude of tail currents of the subtracted current (like those in Fig. 3D) produced immediately upon stepping back to −40 mV after the end of the pulse. A small current was present at the typical resting membrane potential, −65 mV to −60 mV, which is characteristic of M channels. All channels appeared to be open by 0 mV, with the majority opening between −40 mV and −10 mV. Using a single Boltzmann model, the activation curve was fitted with a half-activation voltage of −24 ± 2 mV, a value consistent with previous reports (Tinel et al. 1998; Tatulian et al. 2001; though the range of reported values varies between −8 to −44 mV) and a slope factor of 8.4 ± 1.6. Similar slope values were reported by Wang et al. (1998), Tinell et al. (1998) and Roche et al. (2002) (and others reported values varying widely between 5.5 and 18.6).

M-currents are typically compared based on the time course of the deactivation current. To investigate this property, we used a standard M-current protocol (similar to Adams et al. 1982) in which the current was activated by holding cells at −20 mV for 1 s, then deactivated by sequential, 10 mV hyperpolarizing steps, and finally depolarized again to −20 mV (see diagram in Fig. 4). The presence of 10 μM XE991 reduced the deactivation current (Fig. 4B) by 37% from its level in control solution (Fig. 4A). The wash did not lead to current recovery (Fig. 4C), and in fact, decreased slightly, as was also seen in Fig. 3C. Again, using the control (Fig. 4A) minus wash (Fig. 4C) subtraction method to isolate the irreversibly blocked M-current, we obtained the subtracted current in Fig. 4D. For deactivation to −50 mV, we obtained a time constant of 165 ± 24 ms (n = 5), a value consistent with previously reported M-current deactivation times (Brown & Adams, 1980; Wickenden et al. 2000). By using the depolarization steps to −20 mV at the end of each pulse, we were able to get another measurement of the activation time constant as being 180 ± 9 ms (n = 5), a value similar to our earlier report of 208 ms.

Two activators of M-current are useful for defining the current. Retigabine, and its functional, but less potent analogue flupirtine, exhibit potential as anti-convulsant drugs because of their ability to activate M-current, and thus reduce excitability (Rundfeldt & Netzer, 2000; Wickenden et al. 2000, 2001; Tatulian et al. 2001; Passmore et al. 2003; Martire et al. 2004). In voltage clamp, applying flupirtine led to an increase in overall current when applied to nodose neurons, as can be seen in Fig. 5. Application of 20 μM flupirtine (Fig. 5B) caused a 20% increase in current from control levels (Fig. 5A) and was readily reversible upon washing (Fig. 5C). By subtracting the control current (Fig. 5A) from the current in the presence of flupirtine (Fig. 5B), we obtained the current that increased due to this drug (Fig. 5D). This current had the characteristic shape and slow activation of M-current and showed saturation above −10 mV. The current–voltage relationship seen in Fig. 5E was compiled from the normalized data of seven cells. It illustrates a 7–14 mV leftward shift in activation in the presence of flupirtine across the entire range in which M-current is activated, −60 to +40 mV, much like that seen in previous studies with flupirtine (Martire et al. 2004; Wu & Dworetzky, 2005) but less than that seen with the more potent analogue retigabine (Wickenden et al. 2000, 2001; Tatulian et al. 2001; Martire et al. 2004). Results obtained for 7 out of 10 cells in the presence of 20 μM flupirtine had an average leftward shift of 10.1 ± 0.9 mV, and 4 out of 7 cells in the presence of 10 μM flupirtine showed a leftward shift with an average of 5.3 ± 0.9 mV. In the remaining six neurons we were unable to resolve changes in the M-current because of small simultaneous changes in the large Kv2.1 component. Figure 5F illustrates concentration–response data points for flupirtine on neonatal nodose neurons. The average current increases during a step to −50 mV from baseline levels at −60 mV were plotted for concentrations between 1 and 100 μM. By using a voltage step at a negative potential where other voltage-gated potassium channels are inactive, we were able to ensure that our measurements were limited to M-current. However, since the amplitude of M-current was so small at this voltage, we were unable to resolve small
changes that may have occurred at concentrations lower than 1 μM. For this reason, it was not possible to determine a single IC50 value.

Pharmacological effects on resting membrane potential

M-currents are unique among voltage-gated K+ currents in neonatal nodose neurons in that they activate at, or slightly more negative than, the resting membrane potential (approximately −60 mV). This property was evident in the activation curve in Fig. 3E, but to further investigate the activity of these channels near the resting membrane potential, we employed current-clamp experiments. Membrane potentials were continuously recorded from cells at rest. Figure 6A and B show the effects of 10 μM XE991 on two representative cells; one with a resting potential of −56 mV (Fig. 6A) and the other with a more negative resting potential of −71 mV (Fig. 6B). Figure 6A demonstrates a slow depolarization when XE991 was applied (20–50 s) and a lack of immediate reversibility upon washing (50–80 s). In 14 cells the mean change was 3.6 ± 0.4 mV (range 1–6 mV). Figure 6B illustrates the absence of any response to XE991 at this very negative membrane potential, indicating that this cell was resting below the threshold for activation of M channels (n = 5). Figure 6C and D illustrate representative examples of the effects of 20 μM flupirtine on a cell with a resting potential of −58 mV (Fig. 6C) and a cell with a potential of −65 mV (Fig. 6D). The effect of flupirtine (20–40 s) in Fig. 6C was a rapid, 6 mV hyperpolarization, that readily reversed upon washing (n = 14, 7.0 ± 0.6 mV, range 5–11 mV). No effect was seen from flupirtine in Fig. 6D, when the potential was below the threshold of M-current activation (n = 3).

Also in current-clamp configuration, we evoked action potentials in cells by injecting depolarizing current pulses (typically 5–20 pA). As seen in Fig. 6F, application of 20 μM flupirtine (20–50 s) caused a hyperpolarization of the membrane potential from −41 mV to −49 mV. This hyperpolarization was large enough that applying the same depolarizing current step (15 pA) was no longer able to evoke action potentials. Similar results were obtained in three other cells. This effect was fully reversible after only 10 s of washing.

In most of the cells, adding XE991 to inhibit M-current caused a depolarization that was not large enough on its own to trigger action potential generation. However, in a subset of neurons when a depolarizing current step was used to elicit an action potential, application of 10 μM XE991 increased the number of spikes produced in

![Figure 4. Effects of XE991 on deactivation of M-current in nodose neurons](image)

Experimental data illustrating an example of deactivation currents evoked by the protocol illustrated in the inset (hyperpolarizing the cells from a holding potential of −20 mV to step voltages from −30 to −80 mV in 10 mV increments for 1000 ms each to deactivate the channels and then depolarizing back to −20 mV for 1000 ms to reactivate the M channels). This protocol was applied in the absence (A) and presence (B) of 10 μM XE991 and followed by a wash-out with bath solution after drug application (C). The irreversible XE991-sensitive current (D) was obtained by subtracting C from A. The inset above D shows the exponential fits (black lines) for deactivation to −40, −50 and −60 mV and activation to −20 mV overlaid on the original traces (grey).
response to the current pulse (Fig. 6G). Prior to application of XE991 (black trace), an 8 pA depolarizing pulse evoked only a single action potential from a cell that was resting at −56 mV. However, after application of XE991, the same pulse elicited four action potentials and the resting level of the cell also depolarized to −45 mV. Similar results were seen in five additional cells where there was an increase from one spike to two to seven spikes and an average change in resting potential of 7.2 ± 1.5 mV (range 3–12 mV).

We tested for differential effects of XE991 and flupirtine, on A- and C-type nodose neurons. Using the presence of tetrodotoxin-resistant sodium current to identify C-type neurons (as outlined by Schild & Kunze, 1997), we distinguished between the two neuronal subpopulations and observed the effect of XE991 and flupirtine on each group. In the presence of XE991, A-type neurons showed a depolarization of 4.0 ± 1.0 mV (n = 6, range 3–8 mV) while C-type neurons depolarized 3.9 ± 0.4 mV (n = 6, range 3–5 mV). Upon exposure to flupirtine, A-type neurons hyperpolarized by 3.3 ± 0.3 mV (n = 5, range 2–4 mV), in contrast to C-type neurons that showed 6.7 ± 1.3 mV hyperpolarization (n = 5, range 4–11 mV).

We also investigated the presence and function of M-currents in neurons at physiological temperature (approximately 35°C) in neonatal cells, as opposed to room temperature (22°C) where our other experiments were performed. In this case, XE991 produced a depolarization of the resting membrane potential that was similar to that seen in neonatal neurons at room temperature (n = 6, 4.33 ± 0.97 mV, range 2–8 mV). Also, flupirtine was noted to have the same hyperpolarizing effect at physiological temperature (n = 9, 7.2 ± 1.1 mV, range 3–13 mV).

Next we tested the effects of XE991 and flupirtine on the resting membrane potential of older neurons (from 4-week-old animals) to assess functional developmental changes that may have occurred. Again, we observed a similar depolarization due to XE991 (n = 5, 5.7 ± 1.1 mV, range 4–9 mV) and hyperpolarization due to flupirtine (n = 7, 6.7 ± 0.9 mV, range 4–11 mV) like that seen in neonatal neurons, indicating the continued presence and function of M-currents in adult animals.

Finally, we investigated the antagonistic effects of these two drugs to confirm that they were both producing their effects through the same channel family. Since slow block

Figure 5. Effects of flupirtine on M-currents in nodose neurons
Experimental data showing current elicited by the protocol (shown above C; an 800 ms pulse from a holding potential of −80 mV to voltages between −100 and +40 mV in 10 mV increments, followed by a step to −30 mV for 150 ms) that was applied in the absence (A), presence (B) and wash-out (C) of 20 μM flupirtine. The flupirtine-sensitive current (D) was obtained by subtracting A from B. The current–voltage relationship (E) was plotted as the voltage step versus the normalized current at 800 ms (near the end of the long pulse) from 7 cells for the control data (■) and data in the presence of flupirtine (□). The flupirtine concentration–response data (F) was compiled from the average current increases during a step to −50 mV from baseline levels at −60 mV for cells in the presence of 1 μM (n = 8), 3 μM (n = 8), 10 μM (n = 7), 20 μM (n = 10), 30 μM (n = 14), and 100 μM (n = 7) flupirtine.
is characteristic of XE991, we tested these antagonistic properties by pre-treating cells with 10 μM XE991 for different lengths of time, then measuring the hyperpolarizing effect of 20 μM flupirtine (Fig. 6E). Increasing the time in XE991 prevented the action of flupirtine, as can be seen by the decrease in the hyperpolarization effect. These data indicate that XE991 and flupirtine do exert their effects on the same channels.

**Effects of other channel blockers**

Although barium non-specifically blocks several K⁺ channels, it has been shown to block M channels as well (Cuevas et al. 1997; Selyanko et al. 1999; Passmore et al. 2003). The application of 5 mM barium reversibly suppressed a current with similar properties to those of the current blocked by XE991 (data not shown).

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**Figure 6. Effect of XE991 and flupirtine on resting membrane potential**

Current-clamp recordings of the effect of 10 μM XE991 on representative cells with resting membrane potentials of −56 mV (A) and −71 mV (B). In A, XE991 was applied between 20 and 50 s while in B, XE991 was applied between 20 and 60 s. Current-clamp recordings of the effect of flupirtine on resting membrane potential are shown for cells at potentials of −58 mV (C) and −65 mV (D). Flupirtine (20 μM) was applied to the cells in both C and D between 20 and 40 s. Fresh bath solution was applied to cells at all times other than the intervals when the drugs were used. E illustrates antagonism between XE991 and flupirtine. The average hyperpolarization resulting from application of 20 μM flupirtine is plotted against the length of time the cells were pretreated with 10 μM XE991 (n = 14, 4, 3, 4, 3 and 3 mV for pretreatment times of 0, 5, 8, 10, 12 and 15 min, respectively). In F, action potentials were evoked by injecting 15 pA of current for 9 s out of every 10 s segment. Flupirtine (20 μM) was applied between 20 and 50 ms. G illustrates an increase in action potential discharge in response to an 8 pA depolarizing pulse in the presence of 10 μM XE991 (grey trace), compared with control levels (black trace).
We also tested the potassium channel blocker tetraethylammonium (TEA) because it has been shown previously to exert blocking effects on M-currents, though to varying degrees depending on KCNQ subunit composition (Hadley et al. 2000). To avoid activating the other TEA-sensitive current, Kv2.1, we used a small voltage step at a negative potential (−60 mV to −40 mV) to evoke a small M-current. We then applied TEA at concentrations of 1 mM and 10 mM (a representative example is shown in Fig. 7). Application of 1 mM TEA (light grey trace) produced a very small change in holding current and the current activated by the voltage step from control levels (black trace), while 10 mM TEA (dark grey trace) completely abolished the slowly activating current. As the current in this range is generally only a few picoamperes, it was not possible to construct a reliable concentration–response curve at the lower concentrations. The main observation is that while 10 mM TEA produced a strong block, 1 mM TEA did not. The latter would be expected to block KCNQ2 homomultimers. Similar results were seen in seven neurons.

The only other slow activating current that might account for the current we observed is carried by erg channels which have also been shown to associate with KCNQ currents (Selyanko et al. 1999; Meves et al. 1999). However, astemizole, an erg/eag blocker (Ulens & Tytgat, 2000; Garcia-Ferreiro et al. 2004), had no effect on the linopirdine- or XE991-sensitive current in nodose neurons. Linopirdine has only a very weak blocking effect on the linopirdine- or XE991-sensitive current in nodose neurons. Linopirdine has only a very weak blocking effect on the linopirdine- or XE991-sensitive current in nodose neurons. Linopirdine has only a very weak blocking effect on the linopirdine- or XE991-sensitive current in nodose neurons.

Supporting evidence for M-current in nodose neurons

Separation of potassium currents in native cells is difficult but essential to understanding the role of specific channels in shaping the cell activity. The unique features of M-current and the availability of blocking and activating agents have allowed us to identify an M-like current in the nodose neurons. The common method of comparison of M-currents is typically the deactivation time constant obtained using an ‘M-current protocol’ (Adams et al. 1982). Our deactivation time constant of 165 ± 24 ms at −50 mV is similar to numerous reported values including those of 150–200 ms (Brown & Adams, 1980), and 146 ms (Wickenden et al. 2000) (also Schnee & Brown, 1998; Tinel et al. 2000; Gamper et al. 2003). Other groups have reported the need to use a two exponential fit on their M-currents where the ‘fast’ value is similar to our 165 ms value, and the ‘slow’ value is of the order of 800–900 ms (Wang et al. 1998; Lerche et al. 2000; Pan et al. 2001).

Activation time constants show the same trend where some activation curves are fitted with a single exponential (Brown & Adams, 1980; Adams et al. 1982; Tinel et al. 2000; Gamper et al. 2003) with values similar to those in the present study and others are fitted using two exponentials in which one of the values is in the 100–200 ms range and the other is either faster or slower (Lerche et al. 2000; Pan et al. 2001). A number of explanations can be proposed to account for differences in reported time constants. Studies in expression systems such as Xenopus oocytes (Wang et al. 1998; Lerche et al. 2000; Main et al. 2000; Schroeder et al. 2000) and Chinese hamster ovary cells (Rundfeldt & Netzer, 2000; Selyanko et al. 2000; Wickenden et al. 2000, 2001; Pan et al. 2001; Tatulian et al. 2001; Hadley et al. 2003; Martire et al. 2004) provide useful information.

**Discussion**

In this work we presented the first report of M-currents, and their molecular correlates, the KCNQ2/Q3/Q5 proteins, in visceral sensory neurons. The cognition-enhancing drugs linopirdine and XE991 blocked a current with activation and deactivation characteristics of M-current. XE991 depolarized the membrane potential when its resting level fell within the activation range of M-current. Finally, we showed that flupirtine, the functional analogue of the anti-epileptic drug retigabine, increased a current with M-like properties in these cells and caused a hyperpolarization in resting membrane potential.

**Supporting evidence for M-current in nodose neurons**

Separation of potassium currents in native cells is difficult but essential to understanding the role of specific channels.
about the basic properties of the channels, but may not be entirely representative of the characteristics of endogenous channels where the specific combinations of subunits that contribute to the current in a particular cell are unknown, as is the role of accessory subunits that may modulate the kinetics (such as the KCNE family studied by Tinel et al. 2000). In addition, it has been shown that KCNQ2 can form functional splice variants with different activation and deactivation characteristics (Tinel et al. 1998; Pan et al. 2001). Finally, our electrophysiological activation studies focused mainly on neonatal sensory neurons. There are reports of developmental changes in expression of specific subunits which can therefore give rise to slight changes in kinetics of M-current (Tinel et al. 1998; Shah et al. 2002; Hadley et al. 2003).

Our data provide two other pieces of evidence supporting the presence of M-current in nodose neurons. The first is the activity of this channel in the range of the resting membrane potential (−50 to −60 mV). M-current is one of the few K+ channels that are active at voltages as low as −60 mV (Adams et al. 1982). Our activation curve data showed that M-current in nodose activates between −70 to −60 mV, with $V_{1/2}$ of −24 mV, and our current-clamp studies also showed a depolarization from the resting membrane potential in the presence of M channel blockers. We not only demonstrated this effect on the resting membrane potential of neonatal neurons, both A- and C-type, but on neonatal neurons at physiological temperature, as well as on adult neurons. This characteristic of the current has important implications for the role of M channels in maintaining membrane potential below the threshold for action potential generation.

The second piece of additional evidence supporting the presence of M-current in nodose cells is the increased activation by flupirtine and the hyperpolarizing effect on membrane potential caused by this drug. Our data in the presence of flupirtine display the characteristic leftward shift in the current–voltage relationship as others have seen with flupirtine and the analogue retigabine (Wickenden et al. 2000; Tatulian et al. 2001; Martire et al. 2004) as well as the ‘saturation’ at higher voltage steps (Tatulian et al. 2001). Furthermore, in current clamp 40/40 neurons, including neonatal, adult and those recorded at 37°C, responded to flupirtine with a hyperpolarization of the membrane potential, adding support to the immunological data which indicates the presence of M-current in all nodose neurons.

Note of caution: use of linopirdine and XE991 to evaluate endogenous M-currents

Using HEK cells expressing Kv2.1, we demonstrated the novel finding that linopirdine and XE991 block Kv2.1 in a concentration-dependent manner, and this observation explained why our blocked current in nodose cells consisted of distinct components. Reports of linopirdine affecting other currents, including delayed rectifier currents, have been published (Schnee & Brown, 1998), but no previous reports have specifically identified Kv2.1. Kv4.3 has also been shown to be blocked by linopirdine and XE991 (Wang et al. 1998). These studies provide cautionary notes for attributing the effects of these drugs to effects on endogenous M-currents in native cells, particularly in current clamp experiments where the action potential duration, after-hyperpolarization and repetitive discharge are compared with and without block.

Subunit composition

KCNQ2, KCNQ3 and KCNQ5 immunoreactivity was present at varying levels among the entire population of nodose neurons, as was the current with the characteristics of the M-current which was present in both A- and C-type neurons. It is not uncommon to see all three neuronal KCNQ subunits present in a neuron (Shah et al. 2002; Passmore et al. 2003). The three proteins associate to form different homomultimers (Q2 or Q5) or heteromultimers (Q2 + Q3, Q2 + Q5 and Q3 + Q5). Various KCNQ subunits can be distinguished from one another by different sensitivities to tetraethylammonium (TEA) (Hadley et al. 2000; Lerche et al. 2000; Shah et al. 2002; Passmore et al. 2003). As the M-current in the neonatal nodose neurons was minimally affected at 1 mM TEA, it is unlikely that the M-like current we observed is composed of many KCNQ2 monomeric channels, which are reported to have an IC_{50} for TEA block of 0.13–0.3 mM (Wang et al. 1998; Hadley et al. 2000; Shapiro et al. 2000; Wickenden et al. 2000; Robbins, 2001). In fact, our TEA data suggest that the subunit composition is primarily KCNQ2/3 since TEA at 10 mM blocked the M-current observed during a step from −60 mV to −50 mV (7/7 neurons). Block of homomultimers of KCNQ3 or KCNQ5 or heteromultimers of KCNQ3/5 would be expected at concentrations much higher than 10 mM (Wang et al. 1998; Hadley et al. 2000, 2003; Lerche et al. 2000; Schroeder et al. 2000; Wickenden et al. 2000; Robbins, 2001). In contrast to our results, the IC_{50} values for the response to TEA among nociceptive dorsal root sensory neurons fell into two groups, 0.2–0.6 mM and 3.9–4.7 mM (Passmore et al. 2003), suggesting mixed expression levels of the various subunits among cells. In superior cervical ganglion neurons, developmental increases in the expression of KCNQ3 and subsequent KCNQ2/3 heteromultimer formation are suggested to account for the shift from a two to one component TEA inhibition curve between P17 to P45 rats (Hadley et al. 2003). Similarly, Shah et al. (2002) report two values for hippocampal cells, an IC_{50} of 0.7 mM, and a second one of 1.4 mM (the latter produced only a partial block). At this time, we cannot rule out that we have missed a small
subpopulation of nodose neurons with a high sensitivity to TEA, reflective of KCNQ2 homomultimers. To what extent is there heterogeneity among neurons within the ganglion? In this study we did not discern clear differences among neurons with respect to the amplitude or kinetics of the M-currents such as we and others have seen for HCN (Ih) and tetrodotoxin-resistant sodium currents in nodose neurons. While there are differences in the distribution of KCNQ subunit immunoreactivity among neurons this is not necessarily reflected in somal currents as previously demonstrated by others. For instance, Passmore et al. (2003) demonstrated that KCNQ5 was present in both large and small sensory neurons although the high TEA sensitivity of the M-current did not support a role for this subunit in the somal current. A similar situation exists in hippocampal neurons (Shah et al. 2002). The role of KCNQ5 in those neurons and in the nodose neurons of the present studies remains unknown. The only data in the present study that suggest there may be differences in the functional expression among the nodose neurons is the wide spread in the current response values to flupirtine at each concentration point of the dose–response curve, especially at the higher concentrations (≥ 20 μM). This observation may indicate that the concentration–response relationship of flupirtine is not merely a single component and that these differences may reflect the differing KCNQ subunit compositions of the cells and potentially distinct flupirtine sensitivities of specific subunit combinations. In fact, 2 of 7 neurons tested at both 1 and 10 μM showed little or no further increase in current at 10 μM as compared with 1 μM, while the other cells tested showed greater increases at 10 μM. This suggests that there may be a subpopulation of neurons expressing channels with subunit combinations that are more sensitive to flupirtine. On the other hand, little information is available on the concentration–response relationship of flupirtine on expressed KCNQ subunits. A controlled expression study of the three neuronal KCNQ subunits may be a way to further investigate the effects of flupirtine, and separate concentration–response curves might be necessary for each combination.

Role of M-current in nodose neurons

In our studies we evaluated the role of the M-current in regulating membrane potential. Although this current is relatively small in relation to other currents present in nodose cells, it is active at the resting membrane potential where, within a very narrow range, the input resistance in the neonatal neurons is high (≥ 1 GΩ; Doan & Kunze, 1999). Therefore, changes in M-current of only a few picoamperes can have a significant effect on the resting membrane potential. The effects of XE991 and flupirtine on the membrane potential support this role. At the level of the soma, membrane stability is essential if the information that is transmitted to the central nervous system from the peripheral receptor is to remain faithful to the stimulus. Presently we have no information on the presence of M-current at the peripheral and central terminals of the sensory afferents where modulation by paracrine and neurocrine factors such as substance P, bradykinin and angiotensin II (for review see Brown & Yu, 2000) may play a role in sensory transduction or in synaptic transmission at the central terminals in the nucleus of the solitary tract. Nor do we know which of the KCNQ subunits we have observed in the soma may be expressed in those regions. There is precedence for selective distribution of KCNQ subunits to non-somal regions.

Potential role of visceral sensory afferents in benign familial neonatal convulsions

KCNQ2, KCNQ3 and KCNQ5 subunits have a relatively restricted distribution; they are primarily confined to the nervous system. They have been shown to be present in the hippocampus (Shah et al. 2002), in various cortical regions, cerebellum (KCNQ2 only), spinal cord (KCNQ2 and KCNQ3) and in dorsal root ganglia (Passmore et al. 2003). Mutations in KCNQ2 and KCNQ3 underlie an autosomal dominant form of epilepsy that appears in newborn infants, benign familial neonatal convulsions (BFNC). In addition to convulsions, symptoms are reported to include apnoea and bradycardia in relation to the seizures (Hirsch et al. 1993; Claes et al. 2004). Although not well studied, in at least one case apnoea is reported to precede the seizure (Lerche et al. 1999). The presence of the KCNQ subunits in sympathetic neurons (Brown & Adams, 1980; Wang et al. 1998), intracardiac neurons (Cuevas et al. 1997) and, now, in visceral sensory neurons focuses attention on a possible role of these subunits in symptoms related to the cardio-respiratory reflexes.

References


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