Identification of channels underlying the M-like potassium current in NG108-15 neuroblastoma-glioma cells

A thesis submitted for the degree of Doctor of Philosophy

University of London

by

Jennifer Kathleen Hadley

Department of Pharmacology

University College London

Gower Street

London WC1E 6BT
Abstract

NG108-15 cells express a potassium current resembling the M-current found in sympathetic ganglia. I contributed to the identification of the channels underlying this NG108-15 current. I used patch-clamp methodology to characterise the kinetics and pharmacology of the M-like current and of three candidate channel genes, all capable of producing "delayed rectifier" currents, expressed in mammalian cells.

I studied two Kv1.2 clones: NGK1 (rat Kv1.2) expressed in mouse fibroblasts, and MK2 (mouse brain Kv1.2) expressed in Chinese hamster ovary (CHO) cells. Kv1.2 showed relatively positive activation that shifted negatively on repeated activation, some inactivation, block by dendrotoxin and various cations, and activation by niflumic acid. It had persuasive differences from the M-like current.

mErg is the mouse homologue of the cardiac ether-à-go-go-related gene channel HERG. Having discovered that the HERG blocker WAY-123,398 partially inhibited the M-like current, I studied mErg1a in CHO cells. mErg1a, as previously reported, undergoes intense inactivation, removed on hyperpolarisation to give a transient increase in current preceding deactivation. I report that it can also produce a sustained current with kinetic and pharmacological correspondences to the slow component of the M-like current.

Blocking the presumed mErg component in NG108-15 left a current very similar to the sympathetic ganglion M-current. The latter was recently suggested to comprise a heteromultimer of KCNQ2 and KCNQ3, two neuronal relatives of the cardiac KvLQT1. I expressed KCNQ2 and KCNQ3 in CHO cells, separately and together. Their kinetics match those of the M-current. The M-current blocker linopirdine blocks these channels indiscriminately, while tetraethylammonium blocks KCNQ2 > KCNQ2/KCNQ3 > KCNQ3. Since the selective blockers linopirdine and WAY-123,398, applied together, eliminate the total M-like current, Kv1.2 and other channels are unlikely to contribute. I conclude that blockers, when backed up by kinetic and expression data, were a particularly valuable tool for identifying M-like current components.
Future directions ............................................................................................................................181
References ........................................................................................................................................183

Additional material not bound in


List of figures

Figure 1.1: The NG108-15 M-like current ......................................................................................17
Figure 1.2: Genetic relatedness of potassium channels (taken from Coetzee et al. 1999) .... 21
Figure 3.1: Conductance of rKv1.2 in mouse fibroblast cells...................................................... 45
Figure 3.2: Activation ts for rKv1.2 ............................................................................................... 48
Figure 3.3: Acceleration of rKv1.2 by repeated activation ............................................................ 50
Figure 3.4: Effects of temperature on rKv1.2 activation ............................................................... 53
Figure 3.5: "Hysteresis" in rKv1.2 suggests complex gating behaviour .......................................... 55
Figure 3.6: Steady-state inactivation of rKv1.2 ............................................................................. 58
Figure 3.7: Deactivation of rKv1.2 from +20 mV ......................................................................... 60
Figure 3.8: Dendrotoxin consistently blocks Kv1.2 ....................................................................... 63
Figure 3.9: Effect of niflumic acid on rKv1.2 activation ................................................................. 65
Figure 3.10: Niflumic acid evokes negligible dendrotoxin-sensitive currents ......................... 68
in NG108-15 .............................................................................................................................. 68
Figure 3.11: Example of an MK2 current ....................................................................................... 71
Figure 3.12: Conductance of Kv1.2 in MK2 CHO cells ................................................................. 73
Figure 3.13: Activation time constants for mKv1.2 ................................................................. 76
Figure 3.14: Deactivation of mKv1.2 currents from +12 mV ........................................................... 78
Figure 3.15: Concentration/response curves on mKv1.2 .............................................................. 81
Figure 3.16: Effect of niflumic acid on mKv1.2 ................................................................................ 83
Figure 4.1: Two examples of mErg1a in CHO cells ....................................................................... 96
Figure 4.2: Separation of NG108-15 M-like current constituents ................................................ 101
Figure 4.3: a direct comparison of mErg1a and the NG108-15 slow current ................................. 104
Figure 4.4: Selectivity and concentration-dependence of WAY-123,398 block .......................... 106
Figure 4.5: Fitting methods for mErg1a and the NG108-15 slow current ........................................ 110
Figure 4.6: Voltage-sensitive de-inactivation of mErg1a and the NG108-15 slow current ... 112
Figure 4.7: mErg1a and NG108-15 slow current kinetics compared ............................................. 114
Figure 4.8: mErg1a activation and deactivation compared ........................................................... 117
Figure 4.9: Dissection and reconstitution of the M-like current .................................................... 121
Figure 4.10: Concentration/response curves for four blockers on the mErg1a current .......... 124
Figure 4.11: Concentration/response curves for four blockers on the NG108-15 slow current ................................................................................................................................................. 126
Figure 4.12: Effects of KCNQ and ERG block on NG108-15 firing ............................................. 130
Figure 5.1: Examples of KCNQ2+KCNQ3 currents ....................................................................... 140
Figure 5.2: Two conductance/voltage curves for KCNQ2+KCNQ3 .......................................... 144
Figure 5.3: Two conductance/voltage curves for KCNQ2 ........................................................... 146
Figure 5.4: Two conductance/voltage curves for KCNQ3 ............................................................ 148
Figure 5.5: KCNQ2+KCNQ3 and the NG108-15 fast current compared .................................. 151
Figure 5.6: Partial current/voltage relationships for KCNQ2+KCNQ3 and the NG108-15 fast current ................................................................................................................................................. 153
Figure 5.7a: Deactivation traces and fits for KCNQ2+KCNQ3 and NG108-15 fast currents 155
Figure 5.7b: Deactivation kinetics of KCNQ2+KCNQ3 and NG108-15 fast currents ................. 156
Figure 5.8: Deactivation as 1/τ for KCNQ2+KCNQ3 and NG108-15 fast currents ..................... 158
Figure 5.9: Selectivity of linopirdine ............................................................................................... 162
Figure 5.10: Concentration/response curves on KCNQ channels ............................................. 164
Figure 5.11: Concentration/response curves on the NG108-15 fast current .............................. 166

List of tables
Table 1: Deactivation time constants for the fitted curves shown in Figure 4.2 ....................... 102
Table 2: IC_{50} values for inhibition of mErg1a and NG108-15 slow current by various drugs 127
Table 3: Block of the NG108-15 fast current and of KCNQ currents by linopirdine, azimilide and TEA .................................................................................................................................................. 167
List of abbreviations and constants

ATP: adenosine 5'-triphosphate

cAMP: cyclic adenosine monophosphate

CHO: Chinese hamster ovary

DMSO: dimethylsulphoxide

DTX: dendrotoxin

ERG: ether-a-go-go-related gene

e: exponential constant, 2.71828...

F: Faraday constant, 96480 C/mol

G: conductance

G/V: conductance/voltage curve

G protein: GTP-binding protein

GTP: guanosine 5'-triphosphate

HEK: human embryonic kidney

I: current

IC_{50}: half-maximal inhibitory concentration

IP_3: inositol trisphosphate

I/V: current/voltage curve

MW: molecular weight

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

R: gas constant, 8.314 JK^{-1}mol^{-1}

SCG: superior cervical ganglion

s.e.m.: standard error of the mean

T: absolute temperature, taken as 293K in this work

TEA: tetraethylammonium

THA: 9-amino-1,2,3,4-tetrahydroacridine

UTP: uridine triphosphate

V: voltage (either a command voltage imposed by pClamp, or a recorded voltage between the recording electrode and the bath solution)

V_{rev}: reversal potential (at which ion flow through channels changes direction)

V_{50}: half-maximal activation voltage (at which half of the available channels are active)

τ: time constant for exponential growth or decay

Z: charge on an ion, +1 for potassium.
## Nomenclature and homologues of potassium channels

The KCN... terminology follows the system supported by the HUGO Gene Nomenclature Committee for human genes, and is being adopted for homologues in other species. For K channels, the gene names begin with KCN. All Kv1 channel gene names have the stem KCNA; all members of the Eag family have KCNH, and so on. Clones I have used are shown in **bold text**.

<table>
<thead>
<tr>
<th>Channel name</th>
<th>Species</th>
<th>Synonym(s), Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kv1.2</strong></td>
<td>mouse</td>
<td>KCNA2 OMIM database*</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>MK2 Pongs (1992)</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>NGK1 Pongs (1992)</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>RCK5 Pongs (1992)</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>RK2 Pongs (1992)</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>BK2 Pongs (1992)</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>RAK Paulmichl et al. (1991)</td>
</tr>
<tr>
<td>Eag (ether-à-go-go)</td>
<td>human</td>
<td>KCNH1 OMIM database*</td>
</tr>
<tr>
<td>HERG (human ether-à-go-go-related gene)</td>
<td>human</td>
<td>KCNH2 OMIM database*</td>
</tr>
<tr>
<td>mErg1a</td>
<td>mouse</td>
<td>London et al. (1997)</td>
</tr>
<tr>
<td>minK</td>
<td>human</td>
<td>I_$\text{sk}$, KCNE1 OMIM database*</td>
</tr>
<tr>
<td>KCNQ family</td>
<td>human</td>
<td>KvLQT1 OMIM database*</td>
</tr>
<tr>
<td><strong>KCNQ1</strong></td>
<td>human</td>
<td>Wang et al. (1998)</td>
</tr>
<tr>
<td><strong>KCNQ2</strong></td>
<td>human</td>
<td>Wang et al. (1998)</td>
</tr>
<tr>
<td><strong>KCNQ3</strong></td>
<td>rat</td>
<td>Wang et al. (1998)</td>
</tr>
</tbody>
</table>

Acknowledgements

This thesis is dedicated to the memory of my mother, Kathleen Maud Hadley, 1919-1999.

I would like to express my appreciation to all members (past, present and visiting) of David Brown's lab and the Wellcome Laboratory for Molecular Pharmacology who have helped in the work described here. Particular thanks are due to David Brown for his efforts in obtaining financial support from diverse sources. As a result, the work described here has been supported by Merck Ltd., Sigma-Aldrich Company Ltd., UCL Graduate School, and the Medical Research Council. I also thank Alex Selyanko for collaboration and discussion, and Sacha Filippov for sympathy and light relief. I gratefully remember the contributions of Brenda Browning, Misbah Malik-Hall and Yvonne Vallis to tissue culture work and advice. Joanne Leaney gave helpful guidance on techniques, and her request to test niflumic acid on Kv1.2 encouraged me to be adventurous with drug experiments. Among other members of the UCL Pharmacology department, Don Jenkinson and Tony Dickenson have provided occasional but valued reassurance, Tina Bashford has mediated splendidly in various administrative problems, and Mike Bovingdon has provided moral encouragement and gas-cylinder expertise.
Preface: the M-current

Neurones owe their electrical behaviour to the currents passed by a wide repertoire of ion channels, including many types of potassium channel. Some of these help to set a resting potential across the neuronal membrane, and some shorten action potentials or prevent their occurrence. The M-current is a voltage-dependent potassium current that contributes to regulating excitability by halting firing after a short burst of action potentials. It was described first in sympathetic neurones, and subsequently in some neuronal cell lines such as the NG108-15 mouse neuroblastoma x rat glioma hybrid line. When I started this work, some suggestions had been made about the molecular identity of the channels underlying this current, but there was no conclusive evidence. Thus, my aim was to identify a molecular substrate for the M-like current in NG108-15 cells.

During the course of my study, important evidence was published relating to the molecular basis of the rat sympathetic M-channel. I used this information for some of my experiments. Of necessity, much of this work was done in collaboration with others, and some of the main conclusions have been published (Selyanko et al. 1999). The illustrative and descriptive material in this thesis is drawn almost entirely from my own results. However, clarity demands the incorporation of some results obtained in collaboration with others, which I have specifically indicated as collaborative results.

Background to the M-current

Potassium currents were first observed by Hodgkin, Huxley and Katz in the 1950s as a component of the conductance evoked in the squid axon membrane by a voltage step (Hodgkin et al. 1952, Hodgkin & Huxley 1952a, b, & c). Progress in establishing the physical basis of such conductances was at first slow, as the concept of a “channel” became established, and methods were found for isolating membrane proteins (Hille 1984, p.354). Recording techniques gradually advanced from the impalement of robust amphibian and invertebrate cells to patch-clamping of delicate mammalian neurones.

Still, for many years, currents could only be characterised in terms of their properties: kinetics, modulation and pharmacological blockade. As Rudy (1988) wrote: “...a proper classification is not possible today. However, with the molecular characterisation of K channels and the advent of new specific toxins and drugs, a classification may become possible.” The most advanced molecular biology cited in Rudy’s 1988 review is from his own group: Xenopus oocytes injected with total rat brain messenger RNA showed mixed potassium current types. So it was just becoming clear that potassium currents were produced by genetically-coded proteins, rather than being some undefined property of cell membrane lipids. The rampant proliferation of potassium channel genes had yet to become apparent.

Functional terminology from this era is still widely used to describe potassium currents in native cells. Thus, when a voltage step is imposed, a curve in the evoked current trace represents the development or fall-off of a current. A potassium current which rises (activates) almost instantly and then falls off rapidly (inactivates) on a depolarising step is termed an A-current. A current that activates more gradually, with or without subsequent inactivation, is recognisable as a delayed rectifier. A current that is proportionally larger at strongly negative membrane potentials, leading to potassium entry rather than exit from the cell, is an inward or anomalous rectifier. A current that appears after an action potential, and can be eliminated by omitting extracellular calcium (or introducing a calcium chelator into the cell), is clearly a calcium-activated current. A current which remains steady at a moderately depolarised potential of −20 or −30 mV, declines with an exponential time-course during hyperpolarising steps, is relatively insensitive to most blockers, and can be suppressed through muscarinic receptors, acquired the name M-current.
Discovery of the M-current
The first M-current was reported in 1980 in the frog (Brown & Adams 1980). Frog sympathetic neurones, voltage-clamped at -30 mV, evinced a steady outward current which relaxed in a time-dependent fashion during hyperpolarising voltage steps. This type of recording protocol, covering a limited voltage range and using negative steps to deactivate the current, has become standard for M-currents. Steps at potentials positive to about -20 mV are not feasible, as these would evoke contaminating delayed rectifier currents. Depolarising steps from more negative holding potentials would activate A-currents and sodium- and calcium currents, whereas these are inactivated during prolonged depolarisation. Because of these constraints, kinetic information on native M-currents covers only part of their activation range, and it has never been clear whether they are capable of inactivation at positive voltages. Brown & Adams (1980) found their M-current relatively insensitive to the delayed-rectifier blockers tetraethylammonium and 4-aminopyridine, but susceptible to inhibition by muscarinic agonists. This and subsequent work suggested that the M-current's physiological function was as a brake on action potential generation by the cell. This brake could be released by cholinergic inputs (Adams et al. 1982). The M-current was subsequently found in mammalian sympathetic ganglia (Constanti & Brown 1981). Superior cervical ganglion (SCG) neurones, being easily accessible, became a standard preparation for M-current study.

Properties of the M-current in sympathetic cells
Constanti & Brown (1981) recorded from cells in isolated rat superior cervical ganglia at 29°C, using sharp microelectrodes. The rat M-current appeared as a standing current at holding potentials of -30 to -50 mV. The current was non-inactivating, with a threshold around -70 mV and half-maximal activation at about -45 mV. Single deactivation time constants were measured from 1-second hyperpolarising steps; the curve of 1/t against voltage was U-shaped (centred on V50), relaxations becoming markedly faster with stronger hyperpolarisations.

The M-current is susceptible to few blockers. The anticholinesterase 9-amino-1,2,3,4-tetrahydroacridine (THA; tacrine) at high concentrations of 0.5-1 mM appeared to inhibit the M-current in SCG neurones (Marsh et al. 1990). Barium blocked M-currents in rat SCG with an IC50 of 300 μM (Stansfeld et al. 1993).
Linopirdine (DuP 996) is an experimental cognition enhancer, capable of increasing acetylcholine release from brain tissue, presumably through potassium channel block. In rat brain preparations, it also enhanced dopamine and serotonin release (Aiken et al. 1996). Two more potent linopirdine analogues, XE 991 and DMP 543, have also been identified (Zaczek et al. 1998). In SCG neurones, linopirdine blocks the M-current directly from the extracellular side without involvement of receptors (Costa & Brown 1997), the IC$_{50}$ being reported as 3.4 μM (Lamas et al. 1997) or 7.0 μM (Costa & Brown 1997).

Neurotransmitter inhibition of the M-current is largely responsible for the increased excitability of ganglionic cells in response to the stimulation of endogenous muscarinic receptors (Brown & Selyanko 1985). However, receptor-mediated modulation of the classic M-current is still only partially understood. Calcium has long been recognised as an effective inhibitor of sympathetic M-channels (Selyanko & Brown 1996). Bradykinin inhibition of the sympathetic M-current appears to be mediated by calcium release resulting from phospholipase C activation (PLC) and inositol triphosphate (IP$_3$) generation (Cruzblanca et al. 1998). Endogenous uridine nucleotide-sensitive receptors share this transduction pathway (Bofill-Cardona et al. 2000).

However, muscarinic inhibition cannot be attributed to calcium (Cruzblanca et al. 1998; del Rio et al. 1999; Bofill-Cardona et al. 2000). Selyanko et al. (1992) provided evidence from cell-attached patch recordings of SCG M-currents that, when muscarine was applied to the membrane outside the patch electrode, channels within the patch electrode area were inhibited. This indicates that a messenger generated in the vicinity of the extra-patch receptors is capable of diffusing (or propagating) to the site of the channels being recorded. Because M-channel activity persisted when muscarine was included in the pipette solution, a local modulation (for example, direct G-protein action on the M-channel) is also excluded.

**The NG108-15 neuroblastoma-glioma hybrid line**

Currents resembling M-currents also occur in tumour cells derived from the sympathetic nervous system. Cultured C-1300 neuroblastoma cells, derived from a mouse abdominal tumour, had been previously noted to retain neuronal characteristics such as neurotransmitter synthesis (Amano et al. 1972) and second messenger production (Hamprecht & Schultz 1973). Marshall Nirenberg's laboratory created the NG108-15 neuroblastoma x glioma hybrid by fusing C-1300 neuroblastoma cells (subclone N18TG-2) with rat glioma cells (C6, subclone C6Bu-1) (Klee & Nirenberg 1974, Amano et al. 1974). Daniels & Hamprecht (1974) described the differentiation of the cells by the application of a
cyclic adenosine monophosphate (cAMP) analogue, and gave a detailed account of the morphology of differentiated cells. Hamprecht (1977) enthused that "...the homogeneity of cultured cell material and its almost unlimited capacity for proliferation allow experiments with large numbers of replica cultures. No wonder one is tempted to call...cells like the neuroblastoma-glioma hybrids, the *Escherichia coli of neurobiology.*"

In 1988, NG108-15 cells were reported to have potassium currents similar to those in sympathetic neurones, including an "M-current" (Brown & Higashida 1988a). The idea of NG108-15 cells as a model for real neurones was further strengthened when Robbins & Sim (1990) sought and found a functional analogue of the action potential-shortening A-current (for comparison, see Marsh & Brown 1991).

**Properties of the NG108-15 M-like current**

While the NG108-15 current resembled the sympathetic M-current functionally, it had certain kinetic and pharmacological differences which led to its being termed "M-like", the term I shall use from now on.

Robbins et al. (1992) found the M-like current to be confined to chemically-differentiated NG108-15 cells, and recorded it 4-9 days after differentiation using the patch-clamp technique. Like the sympathetic M-current, the NG108-15 M-like current manifested as a standing current at -30 mV, with increasingly rapid relaxations on stepping to progressively more negative potentials. Working at 35°C, Robbins et al. (1992) found this relaxation to be bi-exponential, but with the faster component of relaxation being more consistent. Like the sympathetic M-current (Constanti & Brown 1981), it had a threshold around -70 mV and half-maximal activation at -44 mV. Its relaxations could be fitted approximately with a single-exponential fit, giving a U-shaped relationship of 1/x to voltage with a minimum around V_n. Nevertheless, the relaxation kinetics were somewhat slower than the sympathetic M-current, despite a higher recording temperature: a (mono-exponential) time constant for the relaxation at V_n was 230 ms, compared with Constanti & Brown's (1981) value of 100 ms.

Figure 1.1 shows a typical NG108-15 M-like current from my own records, and a mouse sympathetic M-current recorded by A.A. Selyanko. The NG108-15 current shows characteristically slow deactivation kinetics, but the voltage-dependence of the tail-currents is clearly similar to that of the sympathetic M-current.
Figure 1.1: The NG108-15 M-like current

The voltage protocol (top) comprises holding at −20 mV and applying hyperpolarising steps, incrementing by −10 mV each time. A 20-40s interval was allowed between steps for the current to recover.

The NG108-15 M-like current (centre) shows slow deactivations, requiring a step duration of 6s to include most of the tail. By contrast, deactivations of the classic M-current (bottom), recorded with a similar but shorter step protocol and shown to the same scale, are complete within a 1-second step. (Mouse SCG M-current record kindly provided by A.A. Selyanko).
NG108-15 M-like current

Mouse SCG M-current (courtesy of A.A. Selyanko)
Pharmacology: potassium channel blockers suggest heterogeneity of “M-currents”

Like the sympathetic M-current, the NG108-15 M-like current was readily blocked by barium (Brown & Higashida 1988a). Robbins et al. (1992) found it to be insensitive to 100 nM dendrotoxin, and comparatively insensitive to tetraethylammonium. It was also blocked by a range of divalent and trivalent cations with, for example, IC$_{50}$s of 2.13 mM for lanthanum and 0.011 mM for zinc (Robbins et al. 1992).

Other channel blockers revealed differences between the NG108-15 M-like current and the classic M-current. The M-like current was inhibited by 9-amino-1,2,3,4-tetrahydroacridine (THA) with an IC$_{50}$ of 8 µM (Robbins et al. 1992). By contrast, the M-current in SCGs was only inhibited at above 100 µM THA (Marsh et al. 1990). Another blocker that discriminates between NG108-15 and SCG currents is a toxin from the scorpion *Buthus eupeus*. 50 µg/ml of the whole venom inhibits the NG108-15 M-like current by 44%, and the SCG M-current by 14% (Filippov et al. 1996). The NG108-15 M-like current is less sensitive to linopirdine than the sympathetic M-current: Noda et al. (1998) measured an IC$_{50}$ of 24.7 µM.

Receptor effects on the M-like current

Early work on the line disclosed that NG108-15 cells were endowed with receptors responding to adrenergic, cholinergic and opioidergic agonists. These gave scope for continuing research using cAMP levels to follow the actions of neurotransmitters (e.g. Traber et al. 1975a, b). NG108-15 cells also synthesize acetylcholine. After several days of exposure to dibutyryl-cAMP, they release acetylcholine in response to various stimuli (McGee et al. 1978). An electrical response of NG108-15 cells to bradykinin application was first noted in 1978 (abstract cited in Brown & Higashida 1988b). Bradykinin application to NG108-15 somata stimulated acetylcholine release from a synapse formed with a muscle cell (Higashida 1988).

The M-like current was inhibited modestly by the action of muscarinic agonists (Brown & Higashida 1988a) and more effectively by bradykinin (Brown & Higashida 1988b). The bradykinin effect could be mimicked by applying phorbol esters or 1-oleoyl-2-acetylgllycerol, implicating PKC in the transduction mechanism (Brown & Higashida 1988c).

Robbins et al. (1992) found no significant muscarinic inhibition of the M-like current in normal NG108-15 cells, attributing this to the absence of M$_1$ or M$_3$ receptors. They therefore studied muscarinic inhibition in a line of NG108-15 stably transfected with the M$_1$ receptor. Although
only PLC-stimulating receptors inhibited M-like currents, no participants in the known signalling pathway (guanosine 5'-triphosphate (GTP), kinases, phosphatases, IP$_3$, calcium) could be conclusively implicated in muscarinic inhibition (Robbins et al. 1993).

NG108-15 M-like currents showed the same “diffusible messenger” phenomenon as SCG M-like currents. That is, channels in a cell-attached patch were inhibited by bath-applied muscarine (Brown et al. 1993). This was confirmed for both muscarine and bradykinin by Selyanko et al. (1995), where thorough analysis of M-like channel data also reveals single-channel conductances centred on 7.5 and 13.5 pS, comparable with sympathetic M-current values (e.g. Selyanko et al. 1992). Another PLC-linked receptor, the P$_{2u}$ or "UTP receptor", is also present in NG108-15 cells and inhibits the M-like current (Filippov et al. 1994).

Potassium channels in the cloning era

It is now common knowledge that potassium channels consist of tetrameric assemblies of protein subunits. Enough potassium channel genes have been sequenced, from bacteria, yeasts, plants and animals, to allow detailed classification and speculation about their evolution (Derst & Karschin 1998). The first potassium channel to be studied was from the fruit-fly Drosophila; it was named Shaker after the effect of a mutation which caused the fly’s legs to shake when it was anaesthetised with ether (Pongs 1992). All the channel subunits I shall deal with in this work are structurally analogous to Shaker, having six hydrophobic transmembrane segments termed S1-S6. S4 contains positively-charged amino acid residues and moves in response to a build-up of positive charge under the plasma membrane, leading to channel opening. S5 and S6 are linked by a peptide chain including a short sequence acting as a selectivity filter for potassium ions. The N- and C-termini of the protein are on the cytoplasmic side of the membrane. These typically contain several consensus sites where phosphate groups can be added by various protein kinases, as well as regions mediating channel assembly as tetramers, the attachment of auxiliary subunits, and interaction with the cytoskeleton.

The first mammalian potassium channels were cloned using the gene sequence of the Shaker channel. As molecular biology identified separate genes for potassium channels, clones were initially named according to their provenance. For instance, Kv1.2 was termed RCK5 (Rat Cortical K channel 5), and NGK1 (Neuroblastoma-Glioma K channel 1) (Pongs 1992). A systematic classification (Kv1.1, Kv1.2, etc.) was gradually adopted, but is now being superseded by a nomenclature based on human gene names (see "Nomenclature and homologues of potassium channels", p.10). Figure 1.2 is a phylogenetic tree showing the
relatedness of mammalian voltage-gated (and calcium-activated) potassium channels, based on homology over the 6-transmembrane region.
Figure 1.2: Genetic relatedness of potassium channels (taken from Coetzee et al. 1999)
Molecular Identification of M- and M-like channels

As previously indicated, the identity of the proteins forming M- and M-like channels was not established when I started work on candidate channels for the NG108-15 M-like current. However, I had several leads resulting from suggestions made by others, which are outlined below and described in more detail in the Introduction sections of Chapters 3, 4 and 5.

(1) Kv1.2. Yokoyama et al. (1989) tried to identify the molecular basis of the NG108-15 M-like current. They isolated two cDNAs: one, which they termed NGK1, coded for the Shaker-related Kv1.2 subunit, while the other, named NGK2, corresponded to the Shaw-like subunit Kv3.1. The latter probably produces the delayed rectifier current found in NG108-15 cells by Robbins & Sim (1990). Kv1.2 shows several differences from the M-like current (elaborated in Chapter 3), but was suggested to form part of the underlying channel, based on its presence in NG108-15 cells and its inhibition by the stimulation of coexpressed M_1 muscarinic receptors in Xenopus oocytes (Huang et al. 1993; Morielli & Peralta 1995).

(2) Ether-à-go-go (Eag) and related genes emerged as contenders during the course of my work. Stansfeld et al. (1997) proposed that a member of the ether-à-go-go family might generate the neuronal M-current. While there are strong arguments against a role in M-currents for Eag itself (Mathie & Watkins 1997; Marrion 1997), sympathetic neurones express cDNAs for several members of the Eag, ERG and Elk channel families (Shi et al. 1997, 1998). Moreover, Faravelli et al. (1996) pointed out the similarity to HERG of an “inward rectifying” current in neuroblastoma-derived cell lines, and Hu & Shi (1997) reported a similar (hence also HERG-like) current in NG108-15 cells.

(3) KCNQ2 and KCNQ3. While I was working on the ERG channel hypothesis, Wang et al. (1998a) advanced strong evidence that the M-channel in sympathetic neurones is formed by a heteromultimer of two recently-discovered members of the KCNQ family, KCNQ2 and KCNQ3.

My work

In the work that follows, I have tried to assess the contributions made by each of the above potassium channel proteins, Kv1.2, ERG and KCNQ2+3, to the M-like current in NG108-15 cells. I have done this mainly by comparing the kinetic and pharmacological properties of the channels, expressed in mammalian cell lines, with those of the M-like current recorded from differentiated NG108-15 cells.
Chapter 2: Methods

Cell culture

CL1023 cells are B82 mouse fibroblasts, stably transfected with rat Kv1.2 and a neomycin-resistance gene (Werkman et al. 1992). They were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 1% foetal calf serum (FCS) and optionally 20 mg/l geneticin (G418), in 50 ml plastic flasks in an incubator at 37°C and 10% CO₂. Cells were subcultured at a dilution of 1:5, 1:10 or 1:20 every 3-6 days as convenient for plating and recording. Subculturing involved a wash with phosphate-buffered saline (PBS) or Hank's balanced salt solution (HBSS), followed by treatment for 2-3 minutes with trypsin-EDTA (containing 0.5g/l trypsin). Once the cells had rounded up, they were dislodged by smacking the flask, fresh medium was added to halt trypsin action, the cells were centrifuged at 1000 r.p.m. for 5 minutes, the supernatant was tipped off, and the cells were resuspended in fresh medium. This suspension was diluted into new flasks, and into a stock solution for plating. Cells were plated in 35 mm plastic dishes, and recordings were made when they approached confluence. The selection medium geneticin (when used) was present throughout the culture cycle, without appearing to have any influence on the properties of the Kv1.2 current. It did not seem to be necessary to maintain expression of the current.

MK2 cells are CHO cells stably expressing the Kv1.2 clone derived from mouse brain, and a neomycin resistance gene. Their culture was similar to that of CL1023, but the incubator was maintained at 5% CO₂. Other differences were that the growth medium comprised RPMI with 10% FCS, 1% L-glutamine (Sigma; giving 2 mM) and 1% penicillin/streptomycin solution (Gibco-BRL; giving 50 units penicillin and 50 µg streptomycin per ml). As a rule, geneticin was added at 100 mg/l. Again, geneticin did not affect measurable properties of the current. Subculture was at 3-4 day intervals.

CHO hml cells are a human muscarinic M₄ receptor-expressing derivative of the CHO-K1 line, described in Mullaney et al. (1993). They were cultured as for the MK2 line, but in MEM alpha medium containing 10% FCS, 1% L-glutamine and 1% penicillin/streptomycin. No selection medium was used. Cells were split at 3-4 day intervals, and channels were introduced by transfection 1-2 days after plating. Recordings were made one or more days after transfection, at an interval determined by experience of the particular current expressed.

Two lines of NG108-15 neuroblastoma-glioma hybrid cells were used: one was an unmodified line, while the other stably expressed pig brain M₄ receptor. These were grown at
10% CO₂ in a growth medium comprising DMEM plus 5% FCS and 6 ml/l HAT supplement (Sigma) giving 3 μM hypoxanthine, 12 nM aminopterin and 480 nM thymidine. Cells were split at a ratio of 1:3 by mechanical trituration, centrifugation and resuspension in fresh medium. Cells were plated in this medium at a 1:6 dilution, on dishes pretreated with a solution of 0.01% poly-L-ornithine (in 0.15 M boric acid adjusted to pH 8.3 with NaOH). Cells were allowed to grow for 24 hours. Chemical differentiation took place in a plating medium containing DMEM with only 1% FCS (to slow the growth of undifferentiated cells) and 6 ml/l HT supplement (Sigma) giving 3 μM hypoxanthine and 480 nM thymidine. This was supplemented initially with 10 μM prostaglandin E1 (PGE1) in DMSO and 50 μM 3-isobutyl-1-methylxanthine (IBMX) in ethanol, to raise cyclic AMP. The cells were left for three days, and then refed with plating medium. Recordings were made 7-21 days after plating, on cells showing neuronal morphology: large somata, processes, and the conspicuous vacuoles characteristic of differentiated NG108-15 cells. A majority of cells in these dishes continued to proliferate and showed a less differentiated morphology, being small and having short or no processes.

As a rule, the kinetic and blocker data shown here were obtained from the unmodified type of NG108-15 cells; the muscarinic receptor-transfected line was used on occasions according to availability. There was no apparent difference in the data from the two lines.

**Transfection**

CHO cells were transfected with channel DNAs using LipofectAmine Plus (Gibco-BRL) according to the manufacturer's recommendations, 1-2 days after plating. Thus, 1 mg DNA was used per dish of cells. This 1 mg was divided into 10 parts DNA for channel(s) and 1 part marker, which was a plasmid coding for the T-cell receptor molecule CD8. All plasmids (supplied by Dr. Ian Wood and Dr. Fe Abogadie) were driven by cytomegalovirus (CMV) promoter. At least one day after transfection, cells for patch-clamping were identified by adding Dynabeads (Dynal (U.K.) Ltd.): microscopic polystyrene beads with a magnetic coating and an anti-CD8 antibody, which binds the bead to cells expressing the CD8 molecule. Dynabeads were first washed by adding 50 μl of bead suspension to 5 ml of pure water, vortexing, placing on a magnetic stand to pull down the beads, and removing the wash water. After resuspending the beads in 1 ml pure water, 0.2 ml of beads was distributed dropwise over each dish of transfected cells. Thus treated, the cells bearing beads continued to survive and proliferate for several days.
Electrophysiology

Dishes of cells were transferred to the stage of a Nikon Diaphot-TMD inverted microscope supported on a pneumatic air-table (Kinetic Systems), and superfused by a gravity-driven system at 10-13 ml/minute. The perfusing solution contained (mM): 144 NaCl, 2.5 KCl, 2 CaCl$_2$, 0.5 MgCl$_2$, 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), and 10 glucose, plus Tris base to pH 7.4.

Patch pipettes

Patch pipettes were pulled on a vertical puller (List or Narishige). Filamented borosilicate glass capillaries (Clark Electromedical) were used. For basic whole-cell recordings, standard-wall (1.5 mm outside diameter; 0.86 mm inside diameter) glass was used to pull electrodes of around 8 MΩ resistance when filled with internal solution. For perforated-patch recording, thin-wall glass (1.5 mm outside diameter, 1.17 mm inside diameter) was used to give controllable final electrode resistances of 2-3 MΩ. The region near the pipette tip was coated with Sylgard (Dow Corning) to reduce capacitance between the electrode and the bath, and the tip was fire-polished on a microforge (Narishige) to facilitate sealing to the cell membrane. Pipettes for whole-cell recording were filled with internal solution using a syringe and a fine cannula. For perforated-patch recording, pipettes were dipped in normal internal solution for about 15s to fill the tip, and then filled up by cannula with internal solution containing the pore-forming compound amphotericin B (Rae et al. 1991). This solution was made daily by dissolving 1 mg amphotericin B in 20 μl dimethylsulphoxide (DMSO), sonicating for 10-20s to dissolve, adding 4 μl per 1 ml of internal solution, shaking and sonicating again. The final amphotericin B concentration was 200 μg/ml, a little weaker than the 240 μg/ml used by Rae et al. (1991).

Considerations relating to internal solutions

Direct electrical recordings from cells involve the use of a pipette solution differing in ionic composition from both the bath solution and the cytosol. This gives rise to two sources of deviation from the transmembrane voltage nominally imposed on the cell. These are the Donnan equilibrium junction potential and the liquid junction potential.

The Donnan equilibrium junction potential arises because, while charge-carrying ions such as potassium and chloride equilibrate between the electrode pipette and the cell interior, there remains a trapped population of cell proteins carrying negative charges. This generates an extra potential difference between the pipette solution and the cell.
interior. If all the intracellular negative charge is fixed, this potential difference can make the cell interior \(-12\) mV negative relative to the pipette, where the electrode wire which measures and maintains the command potential is located. This can be balanced to some extent by having non-diffusing anions replacing chloride in the pipette solution (Horn & Marty 1988; Marty & Neher 1995, p.49). In the case of whole-cell recording, the less mobile anions gradually diffuse between the cell interior and the pipette solution, discharging the potential difference. With perforated-patch recordings, however, a stable potential difference is generated, which will affect the apparent activation range of a current, but not its reversal potential. The Donnan potential can be measured approximately during whole-cell recording by monitoring the shift in a current’s activation curve (Marty & Neher 1995, pp. 49-50). In the case of M-currents, which suffer from rundown in the whole-cell configuration, such measurements are not feasible, and it can only be assumed that measured kinetic parameters may be displaced by up to 12 mV positive to their true positions.

While the Donnan potential arises from immobile ions, the second source of error is determined by the rates of movement of mobile ions, and can be measured and corrected without reference to current properties. A liquid junction potential is a potential difference across the boundary between two solutions. Because the electrode potential is typically standardised by setting to zero when immersing the pipette in the bath, the junction potential between the pipette- and bath solutions is made invisible to the recording apparatus. When the pipette solution abuts the cytoplasm of the cell being patched, this potential difference is no longer present, but the quantity is still being subtracted instrumentally.

If the pipette/bath junction potential is measured, a retrospective correction can be made to the command voltages imposed on the cell. The larger (less mobile) the ions in the pipette solution, the more slowly they diffuse into the adjacent bath solution, and the bigger the potential difference that builds up between the two solutions. Thus, pipette solutions rich in mobile chloride ions create only a small junction potential, but those containing high proportions of acetate generate a significant junction potential.

For Kv1.2-expressing cell lines, I made liquid junction potential measurements in current-clamp mode on the Axoclamp-2, and the same bath (ground) electrode used for recording in each case (see Electrodes section, p.29). I immersed a low-resistance (1-3 M\(\Omega\)) electrode in a bath perfused with the pipette solution, zeroed the pipette potential, changed to the standard external solution, and waited until the voltage readout settled at
a stable value. When I obtained two or three measurements in good agreement, I took an average as the junction potential correction to be applied to the command voltage.

For the internal solution used with mErg1a, KCNQ channels and NG108-15 cells, I measured the liquid junction potential using the Axopatch-200 and the agar-bridge ground electrode arrangement. I immersed an electrode (2-3 MΩ) in a bath perfused with the pipette solution, set the amplifier to voltage-clamp mode, and used the seal test function in Clampex to zero the electrode potential and check the electrode resistance. With the potential set at 0 mV, I then closed Clampex, switched the Axopatch-200 to current-clamp mode, changed the perfusing solution from the pipette solution to the external, and waited for the voltage reading to stabilise. As before, I calculated an average value.

**Compositions of internal solutions**

Early CL1023 recordings were made with an internal solution based on that used by Beech et al. (1991) and intended to approximate to background cell calcium-buffering activity. Its composition was (in mM): 175 KCl, 5 HEPES, 0.1 tetrapotassium salt of 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), 3.3 MgCl₂, 3 Na₂ATP, 0.3 GTP, and NaOH to pH 7.2. The pipette/bath junction potential is expected to be low for a chloride-based internal; I measured a value of less than −2 mV, so I have not made any adjustment to voltages in my results for CL1023 cells.

Experiments to improve the recording lifetime of CL1023 cells used an acetate-based internal solution, based on Robbins et al. (1992, 1993). Dendrotoxin experiments on CL1023 were done with a solution of (mM) 130 K acetate, 20 KCl, 40 HEPES, 3 MgCl₂, 3 ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), and NaOH to pH 7.2.

The formulation chosen for the MK2 line contained (mM): 90 K acetate, 20 KCl, 40 HEPES, 3 MgCl₂, 3 ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), 3 Na₂ATP, 0.3 Na-GTP, and NaOH to pH 7.4. An alternative version with the addition of 1 mM CaCl₂ caused such instability of Kv1.2 recordings that calcium was not routinely added. Selyanko et al. (1995) obtained a junction potential of −4 mV for a similar solution against the standard external solution. I established a junction potential of −8 mV (n=2 identical values) for this solution against the standard external, and I have used this to adjust all my results on the MK2 line.
For recording NG108-15, mErg1a and KCNQ channel currents, the pipette solution contained (mM): 80 K acetate, 30 KCl, 40 HEPES, 3 MgCl₂, 3 EGTA, 1 CaCl₂ and NaOH to pH 7.4. The amphotericin B perforated-patch technique (Rae et al. 1991) was used for all these experiments. The junction potential should be slightly lower than that for the acetate-based solution used on mKv1.2, because of increased pipette [chloride] relative to [acetate]. The measured value was −7.3 mV (n=4), but I have left my NG108-15, mErg1a and KCNQ data uncorrected to maintain consistency with our published reports.

Drug addition
All drugs were applied cumulatively, by addition to the reservoir of perfusing fluid. Niflumic acid was applied as a stock solution in dimethylsulphoxide (DMSO), prepared on the day of the experiment. Forskolin and dideoxyforskolin were dissolved in DMSO and subdiluted in water. Linopirdine stock solutions were made up in 0.1N hydrochloric acid, taking care not to disturb the pH of the perfusing solution by adding more than 0.1% of the acidic solution. Concentration/response curves for linopirdine were completed by washing off the drug and starting again with stronger stock. All other drugs were dissolved in deionised water, sonicating to dissolve if necessary.

Hardware and software

Electrodes
The bath (ground) electrode for CL1023 experiments consisted of a chloride-coated silver wire, connected to the back of the amplifier headstage and positioned in the dish of cells. Experiments on MK2, CHO hm1 and NG108-15 cells used an agar bridge filled with 3M KCl to connect the dish of cells to a pool of KCl contacting a silver/silver-chloride pellet connected to the headstage.

The recording electrode was a chloride-coated silver wire mounted in a Perspex holder with an outlet for suction tubing, and connected to the amplifier headstage. Electrode pipettes, once filled with sufficient internal solution to immerse the tip of the wire, were inserted over the wire, and the assembly was manoeuvred over the dish using a mechanical micromanipulator (Goodfellow Technology).
**Instrumentation: (1) Axoclamp-2**

For discontinuous voltage clamp and current-clamp experiments, an Axoclamp-2 amplifier (Axon Instruments) was used. This received an input signal from a computer (Dell) running Clampex 5 (for early CL1023 recordings) or Clampex 6.0 versions (software from Axon Instruments), via a TL-1 analogue-to-digital converter (Axon Instruments). The voltage output of the amplifier was connected through an oscilloscope to a pre-amplifier and thence to the interface. The current output was connected through another pre-amplifier to the oscilloscope, and in turn to the interface. The Monitor and Sample clock outputs of the amplifier were connected to a second oscilloscope (used for making instrumental adjustments and monitoring recording). A pen recorder (Gould 2400S) received voltage and current signals out of the first oscilloscope. Additionally, a pulse generator for generating test pulses for sealing was connected to the Axoclamp-2, the interface and the first oscilloscope.

**Recording procedure with the Axoclamp-2**

Once the pipette tip was below the surface of the bath solution, the pipette potential was zeroed using the Input Offset knob. Next, -11.1 nA pulses (15 ms duration) were applied from the pulse generator, giving an approximately square downward-going voltage wave on the first oscilloscope (Tektronix). The Bridge control on the Axoclamp was used to level the oscilloscope trace, giving a readout of the electrode resistance. The electrode was positioned just above the chosen cell, and lowered carefully until the pipette made contact with the cell membrane and the trace dipped, indicating increased resistance. Suction was applied through a fine tube connected to the electrode assembly. When the “Vm” readout on the amplifier reached approximately -20 mV, the pulse size was reduced to -1.1 nA. A combination of extra negative current (applied via the DC current command knob) and further suction achieved a seal. Seal resistance could be estimated from the “Vm” and “I” readings on the amplifier, using Ohm’s law. \( V=IR \). \( R = \frac{V}{I} \), thus a seal in the GΩ range (10⁸ Ω) was indicated when (say) an extra applied current of 0.1 nA (10⁻¹⁰ A) caused a change of 100 mV (10⁻¹ V). These applied currents and voltages sound drastic, but worked reliably.

At this point, either additional suction was used to rupture the patch of membrane enclosed by the pipette tip, so going whole-cell, or amphotericin B was allowed to permeabilise the membrane by waiting for several minutes. The trace on the oscilloscope then returned to a shallow trough shape, indicating a lowered resistance.
Switching to direct current clamp (DCC) mode, the capacitance neutralisation knob was used to square off the trace on the second oscilloscope (Telequipment). The Holding Position knob could be used at this point to balance the resting membrane potential of the cell and obtain a readout. A suitable holding potential was then set.

Switching to single-electrode voltage clamp mode, the sampling rate was increased from around 3 to 6.2 kHz to get three cycles on the second oscilloscope. The gain was turned up progressively as high as possible (routinely 8-25 nA/mV) by alternate adjustment of the gain and capacitance neutralisation knobs, to obtain the maximum amplitude of traces without oscillation. This constituted the optimal amount of feedback to achieve the voltage specified by the command from the computer. Clampex version 6 enabled simultaneous recording of both current and voltage traces with the Axoclamp. This showed that good clamp was regularly obtained: the voltage traces were square, and (as an example) a command to a nominal +60 mV achieved nearly +50 mV even when the evoked current was around 10 nA. Thus, current activation kinetics were probably well represented, and current amplitudes could be plotted against actual voltages achieved.

Some recordings on NG108-15 cells (see mErg1a section) were made in current-clamp mode on the Axoclamp. There were only minor differences from voltage-clamp experiments. The signal from the computer was routed through the interface to the EXT. ME1 COMMAND (rather than EXT. VC COMMAND) connection on the Axoclamp. The Axoclamp DESTINATION knob was set to ME1 (rather than VC), and the instrumental adjustments went only as far as those carried out in DCC mode. A small current command was applied as necessary to maintain a membrane potential of -90 mV. Rather than break the circuit to switch to voltage-clamp mode, I have assumed that the properties of the M-like current in these cells were typical.

Instrumentation: (2) Axopatch-200 recording procedure

The apparatus used for continuous voltage clamp was an Axopatch-200 amplifier (Axon Instruments), for some recordings a EF5-01 lowpass/highpass filter (Barr & Stroud) set to 1 kHz, chart recorder (Gould), interface and computer as above.

Recordings on the Axopatch-200 were made as follows. Once the pipette tip was below the surface of the bath solution, the pipette potential was zeroed using the Pipette Offset knob. Pulses of -0.7 mV were applied using the TestSeal command in Clampex. This gave an approximately square downward-going current wave on the computer screen. The electrode resistance could be read out on screen. The electrode was lowered
carefully towards the chosen cell, until the pipette made contact with the membrane and the square trace became shallower, indicating increased resistance. Suction was applied to the electrode assembly, and the seal resistance was monitored on screen. When it reached 30-40 MΩ, additional negative voltage was applied using the keyboard control. Once a value approaching 1 GΩ was attained, test pulses were switched off and the amphotericin B was allowed to permeabilise the membrane by waiting for several minutes. The progress of current development was observed on the amplifier current window and the Gould chart recorder.

**Compensation of series resistance**

With the Axoclamp-2, capacitance and gain adjustments were an intrinsic part of recording, and success in achieving the command voltages could be evaluated from recordings of voltage simultaneously with current. With continuous single-electrode voltage-clamp, the only voltage-clamp option with the Axopatch-200, adjustments are not automatic. I did not routinely use capacitance/resistance compensation. It was assumed during collaborative work on the M-like current that, as most NG108-15, mErg1a and KCNQ currents were less than 1 nA at −20 mV, errors would not be large. The error in voltage achieved is given by $I.R_a$ where $I$ is the total current and $R_a$ is the access resistance. Access resistance in a well-perforated patch can be assumed to be ≥3 times the original resistance of the electrode when immersed in the bath solution. I used electrodes of 2 MΩ or slightly more. A 2 MΩ electrode recording a current of 1 nA “loses” around 1 nA x 6 MΩ = 6 mV across the electrode-cell contact. So, for the KCNQ2+KCNQ3 currents I recorded with the M-current protocol, which averaged 1 nA when holding at −20 mV, the maximum error should be below 10 mV. Average maximal mErg1a current amplitudes, even when boosted by de-inactivation, were below 1.7 nA, giving the same order of error. The NG108-15 M-like current amounted to 0.5-1.5 nA at −20 mV, so the expected errors for this current are similar to those for the cloned currents in CHO cells.

At the more positive voltages used to evaluate KCNQ2+KCNQ3 activation properties, errors are larger. A 2.5 MΩ electrode recording a current of 3.5 nA at a nominal +50 mV gives rise to a voltage error of 3.5 x 7.5 = 26 mV, so the actual current properties reflect those at about +20 mV. I have included some such large currents in my calculations of KCNQ2+KCNQ3 activation curves, but the average maximum current size is around 2.3 nA at a nominal +50 mV and 1.6 nA at the +20 mV point I have used for normalisation. I therefore estimate an average maximum error of about 20 mV for the most positive
potentials used in activations of KCNQ2+KCNQ3 currents. KCNQ2 expressed separately produced currents of similar size, so similar errors are expected. For KCNQ3 expressed alone, the small currents (average 262 pA at +20 mV) are unlikely to be affected by this problem.

This type of shortfall in applied potential will not affect activation thresholds and reversal potentials, where there is little current, but can lead to inaccurate values for parameters such as the half-activation voltage. However, these can also be affected by inactivation when the true command potential is approached.

I subsequently made some recordings of large KCNQ currents incorporating capacitance and series resistance compensation as follows. After ascertaining with a series of voltage steps that the current properties were satisfactory, filtering was bypassed by setting the amplifier's intrinsic lowpass filter to 50 kHz. Using a test pulse from Clampex, at −60 mV (outside the activation range of the current), the Whole Cell Capacitance and Series Resistance knobs were adjusted until the initial transient was eliminated. The Series Resistance Compensation "prediction" knob was turned to 95%, and the "correction" knob was carefully turned as high as possible, reducing the Series Resistance setting to remove persistent transients as they appeared on the test pulse on-screen. I seldom managed to compensate by more than 70-80% without substantial decline in the currents (with or without a resistance drop suggestive of going whole-cell). The Axopatch filter was reset to 1 kHz for recording. Adequate capacitance compensation was verified by eye, using the capacity transients visible on voltage steps.

Series resistance compensation increased the amplitude of KCNQ currents at −20 mV, indicating that an improved (more positive) voltage was attained. It also revealed a small amount of inactivation in KCNQ2 and KCNQ2+3 currents at high positive potentials. The scale of this inactivation is shown in Selyanko et al. (2000): inactivation of KCNQ2+KCNQ3 currents at +50 mV is removed immediately on hyperpolarising, leading to a small increase in current on the first few steps negative from +50 mV. KCNQ3 current inactivation was obvious even without compensation.

Overall, therefore, I consider series resistance errors to have little impact on the NG108-15 M-like current and the candidate mERg1a and KCNQ2+KCNQ3 channels when recorded with the M-like current deactivation protocol. Activation properties measured on KCNQ2 and KCNQ2+KCNQ3 currents over a wider range of potentials, however, are approximate rather than definitive.
Analysis

Initial data analysis was done in Clampfit versions 5, 6 or 8 (Axon Instruments). Results of fitting and amplitude measurements were exported to Quattro Pro (Borland, version 5.0) or Microsoft Excel 97. Spreadsheet calculations and data records for illustration were exported to Microcal Origin (version 4.1 and 5.0), which was also used to fit conductance/voltage and concentration-response curves.

Leak-subtraction of data records

Before evaluating current amplitudes for current-voltage and conductance-voltage curves, records were usually leak-subtracted. In Clampfit 6, cursors were positioned near the end of the current step, and a set of Basic Statistics obtained. The mean amplitude between the cursors was plotted as a quasi-steady-state current/voltage (I/V) curve. A straight line was fitted to the linear portion of this, negative to the activation threshold of the channel, and the slope in pA/mV was noted from the Results table. The data file was then re-opened, and the leak adjusted using the reciprocal of the slope. An alternative method (used with Clampfit 8) was to export the I/V measurements to Excel and then use Excel’s AutoFill feature to create a leak line from sub-threshold data points. This allowed greater selectivity, as any three (or more) well-aligned points from -110 to -60 mV could be chosen, omitting less trustworthy points. The resulting set of extrapolated leak values was subtracted from the raw measurements.

Fitting deactivation tails

The time constant $\tau$ is the time for the current to fall by 63% of its initial magnitude ($1-1/e$). Fits are only trustworthy if the fitted region occupies a time $\geq \tau$. 86% ($1-1/e^3$) of the current relaxation occurs within two time constants, 95% ($1-1/e^5$) within three, and 98% ($1-1/e^7$) within four.

For NG108-15, mErg1a and KCNQ currents, accurate measurement of the deactivation tails in Clampfit required back-fitting of exponential curves. This served to exclude capacity transients, and the brief rising phase of mErg1a currents as they de-inactivated at the beginning of the voltage step. Where multiple current constituents were present in NG108-15 cells, three exponential components were fitted, with the first fitting cursor at the earliest feasible position and the second cursor as late as possible in the step. The fastest component, $\tau \sim 50$-100 ms, was taken as representing the KCNQ-like current, and
the two slower components with τs of -100-800 ms and -1000-2000 ms were attributed to the mErg1a-like current. Expressed mErg1a currents in CHO cells were fitted with two exponential components. The residual fast current present after WAY-123,398 block of the NG108-15 slow fraction was fitted with one exponential. KCNQ currents expressed in CHO cells, however, gave visually better fits when two exponentials were fitted.

To standardise the amplitudes corresponding to these time constants, two methods were available. One involved isolating the fit curves calculated by Clampfit, and refitting the fit curves with the fitting cursor repositioned at the first sample of the voltage step. Alternatively, the fitted amplitude was manipulated using a calculation based on the exponential equation. $I_t = I_0 \cdot \exp(-t/\tau)$, where $I_t$ is the fitted current amplitude (relative to the fitting offset) at time $t$, while $I_0$ is the current amplitude at time zero (i.e. the start of the voltage step). By rearrangement, $I_0 = I_t / \exp(-t/\tau)$. This equation was used on the exported Clampfit Results Table fit data in the spreadsheet program, using Amplitude #1 as $I_t$, (Start Fit – episode start time) as $t$, and Tau #1 as $\tau$ (and similarly for Amplitude #2, Tau #2, etc.). Start Fit is the position of the first fitting cursor. The time of the first sample in the voltage step (episode start time) could be verified as follows. In Clampex, the number of samples in the first holding interval, plus any steps preceding deactivation, plus 1, gives the sample number corresponding to the start of the deactivation step. In Clampfit version 6, the cursor position readout gives both the sample number and the time in milliseconds, so locating the active cursor on this first sample reveals the episode start time.

Adjusted amplitudes obtained by re-fitting fitted curves, and by calculation, matched very closely (commonly to at least 5 significant figures), validating both methods.

Where only one current was present, as in transfected CHO hm1 cells, Clampfit version 6 allowed a quicker method of obtaining the total back-fitted deactivation amplitude for kinetic and concentration-response analysis. Clampfit's Cursor #3 was positioned on the first sample of the voltage step (determined as above). Fitting cursors were positioned, avoiding transients, and the requisite number of exponential components was fitted. The exported Results Table includes the back-fitted current amplitude as Cursor #3 Intercept, and the level of the asymptote to which the curve will eventually fall as Offset. The total deactivation amplitude is obtained simply by subtracting one from the other.
More detail on fitting equations

In Clampfit, I fitted standard exponential growth and decay functions to raw current data, using the Chebyshev method for speed. I did preliminary analysis of time constants, current amplitudes, conductances and inhibition by blockers in Quattro Pro or Excel. I used Origin to graph the results and fit Boltzmann and Hill functions, using the least-squares method.

Normalised current/voltage (I/V) and conductance/voltage (G/V) plots were fitted with the Boltzmann equation:

\[ \frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp\left(\frac{V - V_{\text{half}}}{k}\right)} \]

or \[ \frac{G}{G_{\text{max}}} = \frac{1}{1 + \exp\left(\frac{V - V_{\text{half}}}{k}\right)} \]

Here, \( I_{\text{max}} \) is the maximal value of the current (often, but not always, the value to which currents were normalised), and \( G_{\text{max}} \) is the maximum value of the conductance. \( V_{\text{half}} \) is the membrane potential, \( V \), at which the current \( I \) (or conductance \( G \)) is half of \( I_{\text{max}} \) (or \( G_{\text{max}} \)). \( k \) is the slope, measured in mV, and quantifies the voltage range over which there is an e-fold change in current or conductance.

For concentration-response data, amplitudes of drug-inhibited currents were normalised to the control current amplitude, and converted to proportional inhibition of the control current. Inhibition was plotted against concentration on a logarithmic scale, and fitted with Origin’s “logistic” equation, which corresponded to the Hill equation:

\[ Y = \frac{Y_{\text{max}} \times x^{n_H}}{x^{n_H} + IC_{50}^{n_H}} \]

Here \( Y \) is the inhibition, and \( Y_{\text{max}} \) is the maximal inhibition. \( Y_{\text{max}} \) was set at 100% unless this was obviously inappropriate to the data. \( x \) is the concentration of blocker, and the \( IC_{50} \) is the concentration at which \( Y \) is half of \( Y_{\text{max}} \). \( n_H \) is the slope (“Hill slope” or “Hill coefficient”), which characterises the channel-blocker interaction. For a simple interaction of a blocker molecule with a single binding site on the channel protein, \( n_H \) is expected to be 1, but I did not constrain it to 1 when fitting. \( n_H \) values of <1 may indicate that two or more types of channel are present, with different affinities for the blocker. \( n_H \) values of >1 suggest co-operativity of binding. This is a recognised phenomenon in receptors for neurotransmitters, where the binding of one agonist molecule facilitates binding of a further agonist molecule. It can be hard to explain in the case of potassium channel block, where it is assumed that a single blocker molecule obstructs the pore of each channel.

The results of data analysis are quoted as mean ± standard error of the mean (s.e.m.). I have made limited use of statistics for comparison of native currents with their presumed
cloned counterparts; statistical analysis would ideally require comparison of equivalent data for a wider range of channels, and with larger sample numbers.

**Chemicals**

WAY-123,398-A-5 was a generous gift from Wyeth-Ayerst Research (Princeton, New Jersey, USA), and azimilide from Dr. A.E. Busch (Hoechst Marion Roussel, Frankfurt am Main, Germany). Linopirdine (DuP 996) came originally as a gift from Du Pont (Wilmington, Delaware, USA); later it was purchased from Research Biochemicals International (Natick, Massachusetts, USA). Alpha-dendrotoxin was provided by Prof. J.O. Dolly (Imperial College, London, UK). TEA was from Lancaster Synthesis (Morecambe, UK), and all other chemicals from Life Technologies (Paisley, UK), Sigma-Aldrich (Gillingham, UK) or BDH Chemicals (Poole, UK).

**Stably transfected cell lines and clones**

The NG108-15 cells derived originally from Nirenberg's laboratory, and reached us from Professor H. Higashida (Kanazawa University, Japan). The Kv1.2-expressing cell line CL1023 was also kindly supplied by Prof. Higashida. The MK2 line was made available by Dr. David Owen (now at CeNeS Pharmaceutical PLC, Cambridge, UK), as a welcome gift from the laboratory of Dr. Bruce Tempel (University of Washington, Seattle, USA). DNA for mErg1a was generously provided by Dr. B. London (University of Pittsburgh, USA), and DNAs for human KCNQ2 and rat KCNQ3 by Dr. D. McKinnon (State University of New York, USA).
Chapter 3: Kv1.2

3.1: Kv1.2 – a candidate for the M-like current?

Sites of expression of Kv1.2

Kv1.2 was initially cloned from rat brain by using Drosophila Shaker as a probe. As the second brain potassium (K⁺) channel, it was described as BK2 (McKinnon 1989). Alpha-dendrotoxin was used to extract the Kv1.2 protein from mammalian brain (Rehm et al. 1989). In-situ hybridisation (detecting mRNA) and immunocytochemistry (detecting proteins) show Kv1.2 to be localised in various regions of the mouse brain (Wang et al. 1994). Using similar techniques, rat hippocampus, thalamus, cerebral cortex and cerebellum show high levels of Kv1.2 (Tsaur et al. 1992, Sheng et al. 1994). Collectively, proteins of the Kv1 subfamily have a complex distribution in rat brain, with clear colocalisations in some types of neurone (Veh et al. 1995). Southan & Robertson (1998) recently revealed the presence of functional α-dendrotoxin-sensitive channels by patch-clamping terminals in rat cerebellar pinceaux (brushlike structures formed by cerebellar basket cells synapsing on Purkinje cells). Dendrotoxin-sensitive potassium currents (Kv1.1, Kv1.2 or Kv1.6) are also present in guinea-pig hippocampus (Simmons & Chavkin 1996). Kv1.2 is therefore a major feature in the electrical repertoire of the mammalian brain.

Dixon & McKinnon (1996) surveyed mRNA abundance in rat sympathetic ganglia. The coeliac and superior mesenteric ganglia lack M-currents, but express Kv1.2 mRNA at higher levels than the M-current containing superior cervical ganglion. Thus, Kv1.2 message in autonomic ganglia correlates inversely with the M-current, although this does not guarantee that Kv1.2 protein levels also do so.

Outside the nervous system, Kv1.2 is present as mRNA and protein in the rat heart (Nakamura & Iijima 1994; Xu et al. 1996; Barry et al. 1995). Kv1.2 mRNA is also abundant in ferret heart (Brahmajothi et al. 1996). Additionally, Kv1.2 occurs in airway smooth muscle (Adda et al. 1996), cultured oligodendrocytes and their progenitor cells (Attali et al. 1997), and in the PC12 cell line derived from a tumour of the rat adrenal medulla, a region developmentally related to sympathetic ganglia (Conforti & Millhorn 1997; Conforti et al. 2000). Canine colonic smooth muscle expresses another variant of Kv1.2, with a protein sequence 90-91% homologous to the known rodent Kv1.2 channels (Hart et al. 1993).
NG108-15 cells: NGK1 and NGK2

Kv1.2 was cloned from NG108-15 neuroblastoma-glioma hybrid cells, and named NGK1; when expressed in *Xenopus* oocytes, it showed delayed rectifier behaviour with rapid activation and very slow inactivation at 0 mV (Yokoyama et al. 1989). An accompanying gene, Kv3.1, was later suggested as the basis of the inactivating current characterised in NG108-15 cells by Robbins & Sim (1990). The discrepancy between the low tetroethylammonium sensitivity of the NG108-15 inactivating current, and the high TEA sensitivity of Kv3.1, might be resolved by invoking heteromultimerisation (Ito et al. 1992; Yokoyama et al. 1993). In support of this, a new single-channel conductance emerged in oocytes co-injected with Kv1.2 and Kv3.1 mRNAs (Shahidullah et al. 1995a, b). Selyanko et al. (1995) measured single-channel conductances in NG108-15 cells. Their slope conductance of about 18 pS for the delayed rectifier agrees closely with Shahidullah et al.’s (1995a) figure of 18.2 pS for the presumptive Kv1.2/Kv3.1 heteromultimer.

Kinetics of Kv1.2

The activation properties of Kv1.2 are covered at greater length in the Discussion (section 3.3). A threshold in the range of −30 to −40 mV is widely reported for Kv1.2 expressed in *Xenopus* oocytes (Hart et al. 1993; Ito et al. 1992; Paulmichl et al. 1991; Sprunger et al. 1996; Stühmer et al. 1989). A rat cardiac Kv1.2 expressed in *Xenopus* oocytes showed an activation threshold of −52 or −57 mV according to the amount of cRNA injected (Guillemare et al. 1992). In the mammalian cell line CL1023, Werkman et al. (1992) observed Kv1.2 currents at potentials positive to −20 mV.

Grissmer et al. (1994) reported a kinetic curiosity: Kv1.2 currents evoked by steps to +40 mV increased in size with repeated activations. They suggested that this was caused by the existence of successive closed states of the channel. Thus, after deactivation, the channels are initially in a closed state capable of rapid opening, but convert over seconds into a "resting" state from which they can open only slowly.

Russell et al. (1994) described the behaviour of Kv1.2 at near-physiological temperatures. At 32-35°C, canine colonic smooth muscle Kv1.2 currents show a more negative activation range and greater inactivation than at room temperature.
**Potassium channel blockers acting on Kv1.2**

Many blockers of Kv1.2 have been reported, the best-characterised being peptide toxins. Charybdotoxin (from a scorpion), dendrotoxin (from the green mamba *Dendroaspis angusticeps*) and mast cell degranulating peptide (from bee venom) are all potent blockers of Kv1.2 expressed in B82 mouse fibroblast cells (Werkman et al. 1992). Alpha-dendrotoxin generally blocks Kv1.2 (in both oocytes and mammalian cells) with half-maximal current inhibition at 2-4 nM dendrotoxin (Werkman et al. 1992; Stühmer et al. 1989; Guillemare et al. 1992). Dendrotoxin block of Kv1-subfamily channels depends on three amino acid residues in the S5-S6 linker of the channel, which also contains the pore loop (Hurst et al. 1991); dendrotoxin effects are summarised in Harvey (1997). Besides charybdotoxin, several other scorpion toxins also block Kv1.2 (Werkman et al. 1993; Grissmer et al. 1994; Kharrat et al. 1996, 1997; Rogowski et al. 1996). Further, several peptides from sea anemone species inhibit Kv1.2 currents (Schweitz et al. 1995; Pennington et al. 1996a, b; Gendeh et al. 1997; Cotton et al. 1997).

The existence of so many Kv1.2-blocking peptides in animal toxins may reflect the prevalence of Kv1.2 in non-CNS regions of mammals. As Veh et al. (1995) describe, Kv1.2 is found in motor and sensory regions of the spinal cord, while Kv1.2 channels accessible to blood-borne toxins are present in the heart and in some types of smooth muscle. Blocking Kv1.2 channels can be expected to contribute to cardiac failure and general paralysis, adding to the poisonous effect of animal venoms.

While several cardiac drugs affect Kv1.2 currents, the Class III antiarrhythmics d-sotalol, E-4031 and MS-551, do not block Kv1.2 (Yamagishi et al. 1993). The experimental cognition enhancer linopirdine (DuP 996) blocks Kv1.2 with an IC<sub>50</sub> of about 50 μM (D.G. Owen, personal communication), which is much higher than its IC<sub>50</sub> of 3.4 μM on the sympathetic M-current (Lamas et al. 1997).

Kv1.2 can be blocked by the polyunsaturated fatty acids docosahexaenoic and arachidonic acid, their ethanolamides, and Δ<sup>9</sup>-tetrahydrocannabinol (Poling et al. 1996a, b). This appears to be a direct block of the channel. In contrast, the reported effects of arachidonic acid and cannabinoids on M- and M-like currents are receptor- or enzyme-mediated. Thus, arachidonic acid increased and then decreased the NG108-15 M-like current amplitude, with a decrease in relaxation time constant (Béhé & Meves 1992; Béhé et al. 1992), apparently through protein kinase C (Schmitt & Meves 1993). Mammalian hippocampal neurones show a native M-current resembling that of sympathetic neurones. This M-current was increased by arachidonic acid (Schweitzer et al. 1990), whose lipoxygenase metabolite leukotriene C4...
was suggested as the final mediator in the somatostatin receptor transduction cascade (Schweitzer et al. 1993; Lammers et al. 1996). Schweitzer (2000) inhibited this hippocampal M-current by activating native CB1 cannabinoid receptors with the agonist WIN-55,212. However, WIN 55,212-2 did not inhibit the M-current in rat SCGs transiently expressing CB1 cannabinoid receptors, though calcium channels were affected (Pan et al. 1996).

**Neurotransmitter modulation of Kv1.2**

The RAK (Rat Atrial K channel) differs by one amino acid from BK2 (Paulmichl et al. 1991). Huang et al. (1993) expressed RAK in *Xenopus* oocytes, and identified a tyrosine kinase-dependent pathway for inhibition of the current following activation of coexpressed M1 muscarinic receptors. On this basis, they identified as Kv1.2 a tyrosine kinase-inhibitable outward current which they obtained (during steps from −70 to 0 mV) in NG108-15 cells. The same group subsequently raised the possibility that “the RAK protein comprises at least part of the M-current channel” (Morielli & Peralta 1995). Huang et al. (1994) also reported a protein kinase A-mediated enhancement of the RAK clone, reproducing the effect of β2 modulation on the cardiac myocyte delayed rectifier.

**Potential complications: heteromultimers**

The ability of channels to form heteromultimers within and between subfamilies of voltage-gated potassium channels might explain differences between cloned and native currents. There is evidence for the association of different types of Kv1 subunits in *Xenopus* oocytes (Po et al. 1993), rat brain (Sheng et al. 1993; Wang et al. 1993; Deal et al. 1994), and bovine cerebral cortex (Scott et al. 1994a; Shamotienko et al. 1997). It is now widely accepted that Kv channels can only heteromultimerise within subfamilies. (Xu et al. 1995; Shen & Pfaffinger 1995; Tang et al. 1998). However, Shahidullah et al. (1995a, b) claimed Kv1.2/Kv3.1 heteromultimerisation, and Chen et al. (1996) reported distinctive current properties when *Drosophila Shaker* and *eag* channel RNAs were injected simultaneously into *Xenopus* oocytes. Thus, Kv1-subfamily channel subunits have an undisputed affinity for their close relatives, but at best coassemble less reproducibly with subunits from other groups. It is conceivable that, in certain cellular environments, a factor such as a chaperone or beta-subunit might allow “incompatible” channel subunits to cohabit.

**Beta subunits**

The “dendrotoxin receptors” cloned from rat and bovine brain contain not only conventional 6-pass transmembrane potassium channel subunits, but also smaller intracellular protein
molecules. The pore-forming proteins are now considered as α-subunits, and the accessory molecules as β-subunits. The Kvβ subunits are intracellular proteins with consensus phosphorylation sites (Scott et al. 1994b), which associate in rat brain with Kv1.2 and Kv1.4 channels (Rhodes et al. 1995). This association is dependent on a defined molecular region of Kv1 family subunits (Yu et al. 1996; Sewing et al. 1996). Kvβ1.2 causes substantial inactivation of oocyte-expressed human Kv1.2 currents, depending on the redox state (Wang et al. 1996c). The Kvβ1.2 subunit may also have a chaperone function (Accili et al. 1997).

Working on the related channel Kv1.5, Uebele et al. (1996) found that its activation range was shifted negatively in mouse L-cells and CHO cells, compared with HEK 293 cells or *Xenopus* oocytes. Northern blotting correlated this with the presence of an endogenous β-subunit, which was cloned from L-cells as mouse Kvβ2.1 and appeared also to be present in CHO cells. In COS cells, Kvβ2 subunits appear to act as chaperones and stabilise Kv1.2 (Shi et al. 1996). Li et al. (2000) provide an explanation of why Kv1.2 channels may not be well expressed in some cell types. They identify a VXXSL motif in the C-terminal region of Kv1.4 channels, which promotes membrane targeting of Kv1-family channels in HEK 293 cells. Kv1.2 has VXXSN in the equivalent position; its sub-optimal expression in these cells can be improved by an N→L substitution in the C-terminal sequence, or by coexpressing Kvβ2 subunits with wild-type Kv1.2.

**Other molecules associated with Kv1.2 channels**

The targeting of Kv1.2 channels to specific cellular regions implies the existence of control mechanisms, both for directing the protein to the appropriate part of the cell (as in the above paragraph), and for keeping it in place when it gets there. Kim et al. (1995), using a yeast two-hybrid screen of a human brain cDNA library, found that the C-terminal region of Kv1.4 associated with three intracellular proteins having characteristic 90-amino-acid sequences known as PDZ domains (Gomperts 1996). Numerous partners for PDZ domain proteins have been suggested, including neuronal nitric oxide synthase (Brenman et al. 1996), NMDA receptors (Kim et al. 1996), sodium channels and G-protein-coupled receptors, as well as potassium channels. These can potentially create synaptic assemblies of receptors, ion channels and enzymes, probably in specific combinations according to the cell type. Kv1.2 was found colocalised with Kv1.1 and PSD-95 at septate-like junctions in cerebellar pinceaux (Laube et al. 1996). In COS cells, however, Kv1.2 did not cluster efficiently with PSD-95 (Kim & Sheng 1996) unless Kvβ2 was present (Tiffany et al. 2000), suggesting that Kv1.2/PSD-95 binding is secondary to surface availability of the Kv1.2 channels. By contrast, the
intracellular location of SAP97, and its ability to prevent channels reaching the membranes of COS cells, may suggest a role in trafficking (Tiffany et al. 2000).

Summary of the case for Kv1.2 as an M-current constituent

The presence of Kv1.2, at least as mRNA, in cells with M-currents and M-like currents is a necessary precondition for a role in generating M-like currents. Kv1.2 message has been found in SCG neurones (albeit at a low level), and in NG108-15 cells. In the central nervous system, M-like currents have been studied only in selected regions, while Kv1.2 is so widespread that a full correlation might find appreciable overlap.

Regarding kinetic properties of Kv1.2, the possible presence of β2-subunits in the CHO and fibroblast expression systems I have used may affect Kv1.2 current properties. The absence of such chaperone/modulatory subunits in other cells, such as NG108-15, might lead to lower surface expression and an altered voltage threshold for activation of Kv1.2. Alternatively, the presence of some other accessory subunit in neuronal cells might change current characteristics.

The pharmacology of Kv1.2 as previously reported does not match that of M- and M-like currents, especially since the latter are insensitive to dendrotoxin. Conceivably, though, post-translational modifications such as glycosylation might operate more extensively in native cells, and interfere with the access of a toxin to a binding site.

Muscarinic modulation of Kv1.2 has been shown, as have other receptor-mediated effects, with some discrepancies between Kv1.2 and M-like currents. A proviso is that cell-type-specific cytoskeletal associations with receptors and kinases, or the presence of a β-subunit obstructing channel phosphorylation sites, might lead to differences in the capacity for modulation by particular transduction pathways.

Thus, while Kv1.2 showed little resemblance to the sympathetic M-current, it could not be completely ruled out as a constituent of the NG108-15 M-like current when I started this work. There was no way to define how broad a range of effects beta-subunits, heteromultimerisation and other cell-specific post-translational modifications might have. Rodent cells expressing rat and mouse clones of Kv1.2 seemed an appropriate model: as mammalian cell lines, they might be expected to approximate more closely than amphibian oocytes to the rodent neuronal cells which express M- and M-like currents.
3.2: Results

**rKv1.2: the CL1023 line**

The rat homologue of Kv1.2 (rKv1.2), cloned from neuroblastoma-glioma cells and otherwise known as NGK1, was expressed reliably by CL1023 fibroblasts. Figure 3.1 illustrates its properties during a series of 1-second steps from −70 mV to progressively more positive potentials. rKv1.2 shows slow, slightly sigmoidal activation starting around −30 mV, and visible time-dependent inactivation during 1-second steps to above +30 mV.

**Conductance curves**

I constructed the rKv1.2 conductance curves in Figure 3.1 by recording currents with the protocol above, leak-subtracting, and measuring the “peak” and end-of-step amplitudes in Clampfit. These are the same measurements made by Werkman et al. (1992). “Peak” values were the maximum amplitudes between cursors enclosing the whole episode; because activation was not complete on the smaller depolarisations, the “peak” value should not be taken to represent a steady-state current amplitude. End-of-step values were the mean amplitudes between two cursors placed close together near the end of the voltage step, at about 1 second after the start of activation.

To convert current into conductance, it is necessary to calculate the driving force on potassium ions at any given potential, and this requires a reversal potential. Theoretically, the 175 mM potassium internal and 2.5 mM potassium external solutions should give a reversal potential \( V_{\text{rev}} \) of −107.3 mV, according to the equation \( V_{\text{rev}} = \frac{\ln([K^+]/[K^+]_o)}{RT/ZF} \). In practice, I obtained a less negative value. I recorded tail-currents using a deactivation protocol (see later) starting at 0, +10 or +20 mV and stepping down by 10 mV steps to −110 mV. When the initial and final amplitudes of the currents are plotted, the point where the “initial” and “final” curves cross is the actual reversal potential of the current in each cell. An average of 8 cells gave a reversal potential of −90 mV, which is the value used here.

The conductances plotted in the lower part of Figure 3.1 are given by the equation \( G = I/(V-V_{\text{rev}}) \), where \( I \) is in amperes and \( V \) is in volts. Conductances are normalised to the highest value in each case, namely +60 mV for the “peak” conductance and +30 mV for the end-of-step conductance. “Peak” and end-of-step conductances are fitted (up to the point used for normalisation) with Boltzmann curves constrained to start at 0 and finish at 1. The slope of the curve is similar for “peak” and end-of-step rKv1.2 currents, 9 and 7 mV, respectively, per e-fold change in conductance. The half-activation voltages measured from these data are +4.5 and −0.2 mV for “peak” and end-of-step currents, respectively.
Figure 3.1: Conductance of rKv1.2 in mouse fibroblast cells

(Top) Activation protocol;

(Upper part) rKv1.2 current family evoked by voltage steps from −70 mV, starting at −90 mV and incrementing by +10 mV intervals

(Lower part) Calculated conductances ± s.e.m., for “peak” and end-of-step currents in rKv1.2. Fitted parameters for Boltzmann equation:

end-of-step (black):

$V_m = -0.22 \pm 0.25 \text{ mV}$
$k = 7.34 \pm 0.22 \text{ mV}$

“peak” (grey):

$V_m = 4.55 \pm 0.29 \text{ mV}$
$k = 9.06 \pm 0.27 \text{ mV}$
Figure 3.1
Activation kinetics

I attempted a fit of activation time constants to compare with the literature data of Paulmichl et al. (1991) for the RAK channel expressed in *Xenopus* oocytes. One problem is the apparently sigmoidal nature of rKv1.2 activation, and another is that the Kv1.2 current starts to inactivate before it has finished activating. I tried two approaches. One involved selecting the apparently exponential portion of the current evoked by depolarisation (avoiding both the very beginning and the region where the current starts to decline on large steps), and fitting a single exponential growth equation. The second method was to fit three exponential components to long depolarising current steps. Typically, this gave two time constants with a positive amplitude (representing current decline by inactivation), and one with a negative amplitude (representing activation) which I plotted. Figure 3.2 shows the results of both fitting procedures. Both methods gave similar activation time constants which decreased with increasing levels of depolarisation, values being 1-3 s at 0 mV, decreasing to 30-50 ms at +50 or +60 mV.

When recorded at room temperature, rKv1.2 activated more rapidly on repeated depolarisations. Figure 3.3 shows this effect on two different cells using different voltage steps. The records in the upper portion of the figure include a deactivation from +40 mV to -10 mV. The superimposition of the tail-currents at -10 mV demonstrates that deactivation behaviour is independent of the preceding activation rate. The lower portion of Figure 3.3 shows a more abrupt activation of the current on the second trace. In all cells tested, the effect had substantially disappeared 20 seconds later.

Inactivation accumulates with prolonged or repeated activation of Kv1.2, and recovery from the inactivated state also proceeds on a time course of many seconds. An interval of at least 20 seconds between activations from rest is necessary both to maintain the full current amplitude and to avoid changes in activation kinetics.
Figure 3.2: Activation $\tau$s for rKv1.2

(Top) $\tau \pm$ s.e.m. for a single-exponential fit to rKv1.2 currents activated from $-70$ mV.

(Bottom) $\tau \pm$ s.e.m., using $\tau$ values extracted from a three-component exponential fit to rKv1.2 currents activated for 6 seconds from $-70$ mV and showing inactivation during the step.
Figure 3-2
Figure 3.3: Acceleration of rKv1.2 by repeated activation

(Top) Activation/deactivation protocol and four pairs of rKv1.2 currents at progressively longer intervals. The black trace is the current activated from rest; the grey trace is the current re-activated after the specified delay. After a delay of 15 seconds, the current traces coincide.

(Bottom) Activation protocol and four pairs of rKv1.2 currents in a different CL1023 cell, at progressively longer intervals. Here, currents are nearly superimposed after a 15-second delay.
Figure 3-3
To check current behaviour when a full step protocol would take too long, such as during drug applications or temperature alterations, I devised a ramp protocol. This is illustrated in the top record of Figure 3.4. Four positive-going voltage ramps were applied at the minimum interval allowed by Clampex. The effects are striking, though not quantitative because of an unknown time-lag in the manifestation of inactivation phenomena. Still, it is clear that the first activation of the current from rest displays a substantially sigmoidal current/voltage relation, whereas subsequent steps show extra inactivation starting below 0 mV. For all such ramp records, I have shown the voltage traces recorded with the Axoclamp-2 amplifier, as evidence that poor clamp is not responsible for the sudden dips in current. Where appropriate, I have annotated the time-scale for the current records with some voltage landmarks, based on the achieved (rather than command) potential.

The temperature series in Figure 3.4 shows that the discrepancy between initial and accelerated activations of current is greater at low temperatures. The first activation from rest has a higher voltage threshold at 20°C than at room temperature, while the accelerated currents have a similar starting-point. The difference between first and subsequent activations appears to be negligible in the physiological temperature range above about 30°C, where all activations occur at similarly negative-shifted potentials and inactivation is pronounced. Thus, accelerated activation on repeated steps is an interesting "fingerprint" for the expressed Kv1.2 channel in a cell line, but is unlikely to be physiologically relevant.

The negative shift in activation on the second ramp is not visible unless the first ramp has reached a voltage more positive than 0 mV, and is maximal after activation at a nominal +50 mV (not shown). This effect, therefore, seems to follow activation at voltages producing visible rapid inactivation. Figure 3.5 shows an intriguing phenomenon elicited by a series of mirror-image ramps: with a small time lag, the rapid inactivation seen at increasing positive voltage is reversed as voltage falls again. Thus, depolarisation induces both activation and inactivation, and hyperpolarisation leads to both deactivation and removal of inactivation.

On the ramp currents of Figure 3.5 (as with the tail-currents in Figure 3.3), current decline on hyperpolarising follows a similar time-course irrespective of the activation history of the channel. This is apparent in the bottom section of Figure 3.5, where I have plotted the first and second ramp currents against the actual membrane potential recorded by Clampex. A hysteresis loop emerges, in which the current/voltage slope during the latter part of the hyperpolarisation coincides for both currents, and parallels that on the start of the second depolarisation, but is displaced to more negative potentials. This shift probably reflects a time-lag in activation/deactivation related to the ramp speed.
Figure 3.4: Effects of temperature on rKv1.2 activation

(Top) Recorded voltage (left) and current traces (right) using four ramp commands at the minimum interval permitted by Clampex. In each record of this figure, traces are numbered on the far right, and the first activation from rest is indicated in grey. Approximate voltage landmarks (dotted grey vertical lines) are derived from the voltage plots on the left. This (top record) is a control record at room temperature (24°C).

(Centre) Voltage (left) and current (right) traces in the same cell, but during perfusion with chilled Krebs solution, reservoir temperature 20°C. The first (grey) current trace activates more positively than in the control record. The subsequent currents have a similar negative shift in threshold to that in the control record, but show less inactivation.

(Bottom) Voltage (left) and current (right) traces in the same cell during perfusion with heated Krebs, reservoir temperature 33°C. All current activations occur more negatively than in the control condition, the difference in threshold is negligible between the first and subsequent current activations, and inactivation is early and pronounced.
Figure 3-4
Figure 3.5: “Hysteresis” in rKv1.2 suggests complex gating behaviour

(Top) Recorded voltage traces for four mirror-image ramps applied with the minimum delay permitted by Clampex. The first trace is picked out in grey. All voltage traces are substantially superimposed, and (despite the large currents evoked) a command of +60 mV achieves an actual recorded voltage of about +50 mV. Notably, there is no evidence for a serious failure of membrane clamp at positive potentials.

(Centre) rKv1.2 currents evoked by the voltage ramps illustrated above. The left-hand side (ascending ramp command) shows typical rKv1.2 behaviour. On the right-hand side, as voltage falls from +50 mV, the dip in current amplitude is temporarily reversed. Finally, all current traces converge as they finish deactivating.

(Bottom) The first (grey) and second (black) current traces from the current recording (above), plotted against the corresponding actual voltage (top). Positive to −10 mV, currents show different relationships to voltage on depolarising and hyperpolarising, suggesting complex gating behaviour. However, at negative potentials, the current response to hyperpolarisation (both traces) parallels the onset of the accelerated (second) current during depolarisation (black). Allowing for a time-lag in channel opening and closure, these portions of the record show similar voltage-dependency, and therefore could represent a single gating transition going in opposite directions.
**Subthreshold Inactivation phenomena**

Figure 3.6 demonstrates that prepulses at −50 to −10 mV, which themselves evoke little current, caused a reduction of the current amplitude on a subsequent positive voltage step. Thus, there is an inactivation process occurring even at −20 mV that can reduce the current amplitude reached during an ensuing depolarisation, without accelerated activation being apparent: activation $\tau$s change by a factor of 2 at most.

**Deactivation**

One feature of Kv1.2 behaviour is quite consistent whatever the history of current activation. Kv1.2, identified as mRNA in NG108-15 cells during a search for the M-current channel, shows deactivations which visually resemble those of M-currents. Figure 3.7 shows an example of deactivations following a step to +20 mV, and plots of the time constants obtained from eight such records, using a two-exponential fit to relaxations in the M-current voltage range. As indicated in the Methods chapter, fitted $\tau$s exceeding the step duration, such as those of two seconds or more plotted in Figure 3.7 (bottom left), are not quantitatively reliable. However, a slow component of current decline is evident, and necessitates a two-exponential fit. A clearly visible, relatively fast deactivation process is responsible for the $\tau$s of 60-300 ms (Figure 3.7, bottom right), which are similar to M-current values (see Chapter 5). The U-shape of both $\tau$V curves is unexplained. I suspect the upturn in $\tau$ at negative potentials is an artefact, possibly arising from fitting errors. A very slow drift in current would produce a large $\tau$, and probably one rather than two components would suffice at highly negative potentials. Another possible explanation is the presence of endogenous channels active at −70 mV and more negative, though fibroblast cell types are generally agreed not to have prominent ionic currents of their own.
Figure 3.6: Steady-state inactivation of rKv1.2

(Top) Voltage protocol, with a 30-second moderate depolarising step followed by a 1-second step to +30 mV.

(Centre) rKv1.2 currents evoked by the protocol above. A small current is evoked during 30-second steps (prepulses) to −20 and −10 mV. The subsequent current activation at +30 mV is annotated (right-hand side) with the voltage command for the preceding 30-second prepulse. It is clear that the current at +30 mV is reduced by inactivation taking place at −20 or −10 mV, although inactivation is not visible during this step.

(Bottom left) A plot of rKv1.2 current amplitude ± s.e.m., at +30 mV, against prepulse potential indicates that some inactivation also takes place during steps to −40 and −30 mV, where negligible currents are evoked.

(Bottom right) A plot of the time constant, ± s.e.m., for activation of the rKv1.2 current at +30 mV shows little dependence on prepulse potential up to −10 mV.
average normalised amplitude at +30 mV

average τ (ms) at +30 mV
Figure 3.7: Deactivation of rKv1.2 from +20 mV

(Top) Voltage protocol used to study deactivation of rKv1.2.

(Centre) An rKv1.2 current family resulting from this protocol. The initial step to +20 mV, evoking a substantial current without pronounced inactivation, is followed by hyperpolarising steps at −10 mV increments.

(Bottom) A plot of $\tau \pm$ s.e.m., using $\tau$ values obtained from a 2-exponential fit to deactivation tails obtained using the above protocol. The large (slow) $\tau$ values indicate a trend, but are not quantitatively reliable (see text). The faster $\tau$s obtained are ~60-400 ms.
Pharmacology

One of the most consistent characteristics of Kv1.2 channels is their binding and blockade by the snake toxin α-dendrotoxin. The only doubt ever raised was by Guillemare et al. (1992), who found reduced dendrotoxin block in *Xenopus* oocytes over-expressing Kv1.2. At the same time, the insensitivity of M-currents to dendrotoxin was a major bar to their identification with Kv1.2. It was thus relevant to check the sensitivity of Kv1.2 to dendrotoxin in the environment of the mammalian fibroblast cell. Figure 3.8 provides examples of raw data obtained with 150 mM K⁺ internal solution (see Methods) and the perforated-patch recording configuration. All inhibitions are calculated on the maximum current amplitudes attained during the step. Currents evoked at a nominal 0 mV are typically about half-inhibited by 3 nM dendrotoxin, as in the top record in Figure 3.8. Mean data for four CL1023 cells (not shown) gave an IC₅₀ of 3.6 nM. I also applied dendrotoxin under conditions enhancing Kv1.2 activation. In the centre section of Figure 3.8, an rKv1.2 current underwent similar block by 3 nM dendrotoxin, whether currents were allowed to return to their resting state (depolarisations at 40-second intervals) or accelerated by frequent activation (5-second intervals). The bottom part of Figure 3.8 shows that, in the presence of niflumic acid (whose effect I describe below), the greatly enhanced current was even more strongly blocked by dendrotoxin than the control current in the same cell. Because of the different extents of inactivation under control and niflumic acid-treated conditions, an exact assessment of differences was not pursued. Thus, Kv1.2 was consistently sensitive to dendrotoxin.

Niflumic acid is a known blocker of chloride channels (reviewed by Greger 1990). Along with related chloride channel blockers, it has also been reported to affect potassium channels, both calcium-activated (Gribkoff et al. 1996) and KCNQ1/minK (Busch et al. 1994a; Busch et al. 1997a; Abitbol et al. 1999). More recently, a niflumic acid action on KCNQ5 was documented by Schroeder et al. (2000). The effect of niflumic acid on Kv1.2 is therefore interesting both for comparison with the effect on other potassium channels, and as a potential source of interference when it is used to eliminate chloride currents from native cells with a mixed complement of ion channels.

I applied a standard chloride-channel-blocking dose of 200 μM niflumic acid while running my usual Kv1.2 voltage protocols. Figure 3.9 shows the effect of niflumic acid on ramp currents, which resembles the effect of raised temperatures. The differential rate of activation between *de novo* and repeated current activations disappears, and the whole activation curve shifts to more negative voltages. This effect is substantially reversed on 3 minutes washout.
Figure 3.8: Dendrotoxin consistently blocks Kv1.2

(Top) rKv1.2 current activated at a nominal 0 mV (from –70 mV), showing that 50% inhibition in this cell occurs at a concentration between 3 and 10 nM dendrotoxin (DTX).

(Centre) rKv1.2 currents activated once every 40 seconds (left) and accelerated by activation every 5 seconds (right), at 0 mV, with and without 3 nM dendrotoxin; both show similar inhibition.

(Bottom) rKv1.2 currents activated at 0 mV in the absence (left) and presence (right) of 200 μM niflumic acid. The activated current (right) is even more intensely blocked than the control by 10 nM dendrotoxin.
3 nM DTX: 35.7% inhibition
10 nM DTX: 70.8% inhibition
30 nM DTX: 86.1% inhibition

0.025 Hz: 3nM DTX, 52% inhibition
0.2 Hz: 3nM DTX, 61% inhibition

200 μM niflumic acid at 0 mV in the same cell
10 nM DTX in 200 μM niflumic acid: 82% inhibition
Figure 3.9: Effect of niflumic acid on rKv1.2 activation

Recorded voltage (left) and current traces (right) using four ramp commands at the minimum interval permitted by Clampex. Traces are numbered on the far right, and the first activation from rest is indicated in grey. Approximate voltage landmarks (dotted grey vertical lines) are derived from the voltage plots on the left.

(Top) a control recording before niflumic acid application.

(Centre) The same cell, 90 seconds after starting perfusion with 200 µM niflumic acid. All current activations begin at the same negative voltage, and inactivation is increased relative to control.

(Bottom) The effect is reversed after 3 minutes washing out.
Figure 3.9
I tested the availability of Kv1.2 current in NG108-15 cells by applying niflumic acid while running a protocol based on Filippov et al. (1994). From a holding potential of −20 or −30 mV, a hyperpolarisation to −60 mV was given in order to monitor the M-like current relaxation, followed by a further step to 0 or +20 mV to evoke the delayed rectifier.

Figure 3.10 shows a cell in which the standing current increase in 200 µM niflumic acid was comparatively pronounced. Dendrotoxin, added in the continuing presence of niflumic acid, had little effect on the enhanced current at −30 mV and the delayed rectifier at 0 mV, and none on the enhanced relaxation at −60 mV.

In total, nine of eleven NG108-15 cells had an increased holding current at −20 or −30 mV in 200 µM niflumic acid, accompanied by an increased relaxation amplitude at −60 mV. The delayed rectifier was not consistently affected by niflumic acid, showing a slight increase or decrease or no change. Four cells (still in niflumic acid) treated with 100 nM dendrotoxin had a slight decrease in the delayed rectifier, but no change in the enhanced current relaxation at −60 mV. Thus, the NG108-15 M-like current included no dendrotoxin-sensitive Kv1.2 current even after niflumic acid treatment.

In two cells, I tested for a contribution of a classic M-current to the niflumic acid effect, by applying the M-current blocker linopirdine on top of niflumic acid. In these cells, 30 µM linopirdine, which would be expected to produce full block of a sympathetic M-current, produced only a fractional change in the niflumic acid-enhanced NG108-15 current at −30 and −60 mV, the size of the reduction being less than the original standing current and relaxation amplitudes. Thus, it appeared that neither the original standing current nor the additional niflumic acid-evoked current included much of the channel type forming the sympathetic M-current (unless niflumic acid blocked access to the linopirdine binding site).

I also considered a calcium-activated potassium current as a possible cause of the increase in standing current, since niflumic acid was stated to enhance currents through cloned Slo channels (Gribkoff et al. 1996). However, five of the eleven cells were tested in calcium-free Krebs solution, and four showed a standing current increase in these low-calcium conditions. The niflumic acid-evoked current in NG108-15 cells therefore remains unidentified.
Figure 3.10: Niflumic acid evokes negligible dendrotoxin-sensitive current in NG108-15

(Top) Voltage protocol used on NG108-15 cells, designed to demonstrate standing current at −30 mV, M-like current deactivation at −60 mV, and delayed rectifier activation at 0 mV.

(Main picture) Currents elicited by this protocol on an NG108-15 cell showing a clear response to niflumic acid. The control trace (black) shows a small M-like current relaxation at −60 mV. 200 μM niflumic acid (medium grey) causes an increase in standing current at −30 mV, with an increased deactivation amplitude at −60 mV. This cell also shows an increase in the delayed rectifier at 0 mV. Only the delayed rectifier increase was reversed by the Kv1.2 blocker dendrotoxin (pale grey).
-30 mV
-60 mV
0 mV

- control
- in 200 μM niflumic acid
- in niflumic acid + 100 nM dendrotoxin

0.25 nA
0.5 s
0 nA

Figure 3-10
**mKv1.2: the MK2 line**

The CL1023 line developed problems with recording stability, but fortunately Dr. Bruce Tempel’s MK2 line of Chinese Hamster Ovary cells was available. This line was stably transfected with the MK2 (mouse brain) clone of Kv1.2, which I refer to here as mKv1.2. An example of a current family is shown in Figure 3.11. The current was essentially similar to rKv1.2 in its activation, deactivation, inactivation and acceleration phenomena. The minor differences may be attributable to the largely acetate-based internal solution, which I chose because it had partially alleviated the instability of CL1023 recordings. Allowing for a measured junction potential correction of –8 mV with this internal solution, the current (when activated from –78 mV) usually began to activate around –20 mV, and had visible time-dependent inactivation above +30 mV.

**Conductance curves**

I constructed conductance curves as for rKv1.2, after making a junction potential correction of –8 mV to the recording voltages and the reversal potential. The 110 mM potassium internal and 2.5 mM potassium external solutions theoretically give a reversal potential of –95.5 mV, according to the equation given previously. The value used here, obtained empirically from a plot of the initial and final amplitudes of 12 tail-currents, is –80 mV (which incorporates the junction potential correction).

I normalised conductances to the maximum in each data set, plotted normalised values against voltage, and fitted them (up to the point used for normalisation) with Boltzmann curves constrained to start at 0 and finish at 1. Figure 3.12 shows conductances for (top) currents activated after a resting period of at least 20 seconds, and (bottom) currents activated 200 ms after the end of a 1-second prepulse to +52 mV, which produced maximal acceleration. The maximum conductance, to which all values were normalised, was at +32 mV for activations from rest, +12 mV for end-of-step accelerated currents, and +22 mV for “peak” accelerated currents. For mKv1.2 activations from rest, the slope of the resulting curve was 7 mV per e-fold change in conductance, and the half-activation voltage was +5 mV, for both “peak” and end-of-step currents. These values are close to those I obtained for rKv1.2 in the CL1023 line: the “peak” rKv1.2 current had a slope of 9 mV and a $V_{1/2}$ of +4.5 mV, and the end-of-step rKv1.2 current had a slope of 7 mV and a $V_{1/2}$ of -0.2 mV.

mKv1.2 currents accelerated by a prepulse had a slope of 6-7, and half-activation voltage – 18 mV; I did not quantify the properties of accelerated currents for rKv1.2.
Figure 3.11: Example of an MK2 current

(Above) Activation protocol;

(Below) mKv1.2 current family evoked by voltage steps from \(-78\) mV, starting at \(-98\) mV and incrementing by \(+10\) mV intervals.
Figure 3-11

The graph shows the current (nA) over time (s) for different voltages: +52 mV, -78 mV, and -98 mV. The time axis ranges from 0.0 to 1.2 seconds.
Figure 3.12: Conductance of Kv1.2 in MK2 CHO cells

(Top) "Peak" (grey) and end-of-step (black) conductance, ± s.e.m., plotted against voltage for mKv1.2 currents activated for 1s from -78 mV.
Boltzmann fit parameters ("peak"):
\[ V_\% = 5.21 \pm 0.52 \text{ mV} \]
\[ k = 7.16 \pm 0.26 \text{ mV} \]

Boltzmann fit parameters (end-of-step):
\[ V_\% = 4.49 \pm 0.53 \text{ mV} \]
\[ k = 7.04 \pm 0.24 \text{ mV} \]

(Bottom) "Peak" (grey) and end-of-step (black) conductance, ± s.e.m., plotted against voltage for mKv1.2 currents following a step to +52 mV.
Boltzmann fit parameters ("peak"):
\[ V_\% = -17.64 \pm 1.80 \text{ mV} \]
\[ k = 6.89 \pm 0.96 \text{ mV} \]

Boltzmann fit parameters (end-of-step):
\[ V_\% = -17.87 \pm 1.55 \text{ mV} \]
\[ k = 6.12 \pm 0.82 \text{ mV} \]
Figure 3.12

Activating from rest
- end (n=25)
- Boltzmann fit
- peak (n=25)
- Boltzmann fit

After a +52 mV prepulse
- end (n=6)
- Boltzmann fit
- peak (n=6)
- Boltzmann fit

Figure 3.12

membrane potential (mV) vs. mean normalised conductance
Kinetics

Activation of the mKv1.2 current was less sigmoidal than that of rKv1.2, and was readily fitted by two exponential components. Figure 3.13 plots the resulting time constants against voltage. Activation ts were about 120 and 980 ms for a step to +2 mV, and 8 and 78 ms at +52 mV, the maximum command potential used when the junction potential was allowed for.

Acceleration of mKv1.2 activation occurred with repeated depolarisations and at raised temperature, as for rKv1.2. An example of this behaviour by mKv1.2 is shown in the upper left corner of Figure 3.16, where it serves as a control for the effect of 200 μM niflumic acid. Acceleration of the current is only partial because this protocol has two successive steps to the same potential rather than a consistent positive prepulse. However, as described for rKv1.2, prompt re-activation of the current causes a faster rise in amplitude and the appearance of pronounced inactivation, which was previously masked by slow activation of the current.

Inactivation of mKv1.2 during strong depolarising steps resembles that of rKv1.2 (compare Figure 3.11 with Figure 3.1).

Deactivation of mKv1.2 is also superficially similar to that of rKv1.2. Figure 3.14 (upper part) shows an example current family, resulting from an initial step to +12 mV and deactivations at −10 mV increments. Deactivation time constants from records of this type (n=13) gave the plots in the lower section of Figure 3.14, of t against membrane potential. The slower deactivation ts, around 100-300 ms, are mathematically sounder than those obtained on rKv1.2 because they fall well within the 1-second duration of the step (see Methods chapter and p.56). Fast ts are 10-40 ms over an M-current-appropriate range of voltages. All ts, slow and fast, for deactivation potentials negative to −30 mV are below about 200 ms, which would not rule out Kv1.2 as an M-like current constituent.
Figure 3.13: Activation time constants for mKv1.2

t values from a two-exponential fit of Kv1.2 activations from -78 mV, plotted separately:

(Above) slow $\tau \pm$ s.e.m.;

(Below) fast $\tau \pm$ s.e.m.
Figure 3-13

- **mean slow τ**
- **mean fast τ**

**membrane potential (mV)**

**activation τ (s)**

**activation τ (ms)**
Figure 3.14: Deactivation of mKv1.2 currents from +12 mV

(Top, centre) Voltage protocol used to study mKv1.2 deactivation, and corresponding current family.

(Bottom left) slow $\tau \pm$ s.e.m., plotted against voltage;
(Bottom right) fast $\tau \pm$ s.e.m., plotted against voltage. Both data sets from 13 cells.
Pharmacology

Figure 3.15 summarises some concentration/response data on mKv1.2 for dendrotoxin, as well as three substances not previously characterised as Kv1.2 blockers. Dendrotoxin blocked the mKv1.2 current with an IC\textsubscript{50} of 4.5 nM (close to the value of 3.6 nM that I found for rKv1.2, see p.62). Forskolin, tested with the aim of reproducing the reported enhancement of Kv1.2 currents by protein kinase A activation, proved instead to block the mKv1.2 current in a concentration-dependent fashion, with an IC\textsubscript{50} of 25 \mu M.

Dideoxyforskolin, an analogue which does not activate adenylate cyclase, also had a blocking effect at 10 \mu M (n=1; not shown). 2 mM dibutyryl cyclic AMP, a membrane permeable cAMP analogue, had no effect (n=3; not shown). Taken together, this supports the non-involvement of a protein kinase A mechanism in the inhibitory action of forskolin. Lanthanum had an IC\textsubscript{50} of 25 \mu M on mKv1.2, for a maximum inhibition of 74% at +12 mV. Zinc, potentially a physiological blocker of neuronal ion channels, had an IC\textsubscript{50} of 0.43 mM on mKv1.2 at +12 mV. Barium (not shown) had an IC\textsubscript{50} of around 0.8 mM averaged over a range of voltages from -8 to +52 mV; there was no great difference in sensitivity between -8 and +52 mV.

Niflumic acid at 200 \mu M affected mKv1.2 in the same way as rKv1.2. This is illustrated in Figure 3.16, using an activation protocol with two successive steps to the same potential. The current elicited at -8 mV is labelled, to demonstrate the increased amplitude and activation rate in niflumic acid compared with the control current. Using this step protocol on 7 cells, I constructed conductance curves for the niflumic acid-enhanced current. These are shown in the lower part of Figure 3.16. The half-activation voltages of -17 ("peak") and -20 mV (end-of-step) in niflumic acid closely resemble the value of -18 mV for both "peak" and end-of-step currents after a +52 mV prepulse (Figure 3.12). Currents elicited by depolarising ramps in 200 \mu M niflumic acid (not shown) showed a negative shift in activation threshold, like that for rKv1.2 but less pronounced; the first mKv1.2 ramp current from rest was not fully left-shifted. Deactivation showed no major changes in the presence of niflumic acid (not shown).

Two other chloride channel blockers, DIDS and SITS at 100-1000 \mu M, did not accelerate or activate the mKv1.2 current (not shown). This supports a mechanism involving a direct interaction of niflumic acid with the Kv1.2 channel, rather than via endogenous chloride channels.
Figure 3.15: Concentration/response curves on mKv1.2
All data points are plotted as mean inhibition ± s.e.m.

(Top left) Alpha-dendrotoxin (n=4)
Hill equation fit parameters:
$Y_{max} = 100\%$
$IC_{50} = 4.47 \pm 0.82$ nM
$n_H = 0.72 \pm 0.08$

(Top right) Forskolin (n=6)
Hill equation fit parameters:
$Y_{max} = 100\%$
$IC_{50} = 24.58 \pm 0.82$ µM
$n_H = 1.10 \pm 0.03$

(Bottom left) Lanthanum (n=7)
Hill equation fit parameters:
$Y_{max} = 74.38 \pm 0.13\%$
$IC_{50} = 25.38 \pm 11.83$ µM
$n_H = 1.04 \pm 0.20$

(Bottom right) Zinc (n=6)
Hill equation fit parameters:
$Y_{max} = 100\%$
$IC_{50} = 0.43 \pm 0.04$ mM
$n_H = 1.23 \pm 0.14$
Alpha-dendrotoxin:
2 steps, 2 ramps
to +22 mV

Forskolin:
n=6, ramps
to +22 mV

Lanthanum:
n=7, steps
to +12 mV

Zinc:
n=6, steps
to +12 mV

Figure 3-15
Figure 3.16: Effect of niflumic acid on mKv1.2

(Top) Voltage protocol with two successive steps to the same potential.

(Above) mKv1.2 current families evoked by this protocol, in the absence (left) and presence (right) of 200 μM niflumic acid. The current at −8 mV is indicated in grey, to demonstrate the kinetic changes caused by repeated activation and niflumic acid. Note that the effect of niflumic acid on the current profile resembles that of prior activation.

(Below) “Peak” (grey) and end-of-step (black) conductance ± s.e.m., against voltage for activations from rest in 7 MK2 cells in 200 μM niflumic acid.
Boltzmann fit parameters (“peak”, grey):
\[
V_n = +16.77 \pm 1.13 \text{ mV} \\
k = 5.50 \pm 0.48 \text{ mV}
\]

Boltzmann fit parameters (end-of-step, black):
\[
V_n = +20.43 \pm 1.41 \text{ mV} \\
k = 6.12 \pm 0.80 \text{ mV}
\]
Conductance for Kv1.2 in MK2 CHO cells in 200 μM niflumic acid

Figure 3-16
3.3: Discussion

Activation and conductance data

I find an activation threshold of approximately \(-30\) mV for rKv1.2 in CL1023 cells, and \(-20\) mV for mKv1.2 in the MK2 line. The half-activation voltage is 0 mV for rKv1.2 measured at the end of a 1-second step, and +5 mV for the "peak" rKv1.2 current (in this case, the highest current measured during a 1-second step). The half-activation voltage is +5 mV for mKv1.2 for both "peak" and end-of-step measurements. Pre-activation of the mKv1.2 current by a large positive pulse caused a shift of the half-activation voltage to \(-18\) mV.

Werkman et al. (1992) observed rKv1.2 currents at potentials positive to \(-20\) mV in CL1023 cells. Their half-activation voltages, 0 mV for the "steady-state" current and +5 mV for "peak" current, were identical to my figures for this cell line. Using the same cells, Grissmer et al. (1994) reported a half-activation voltage of +27 mV for the "peak" conductance.

Kv1.2 has often been expressed in *Xenopus* oocytes. The activation threshold in this cell environment is generally reported to be somewhere negative to \(-30\) mV. Stühmer et al. (1989) report a threshold negative to \(-40\) mV for rKv1.2 (RCK5 clone) in oocytes, with a half-activation voltage of \(-34\) mV. Ito et al. (1992) give the threshold for rKv1.2 (NGK1) activation in oocytes as \(-36\) mV. Hart et al.’s (1993) *canine colonic Kv1.2* produced currents at \(-30\) mV in oocytes. rKv1.2 (RAK clone) in oocytes began to activate positive to \(-40\) mV (Paulmichl et al. 1991). Sprunger et al. (1996) also worked on RAK, observing that substantial activation occurred around \(-35\) mV; their activation curve (from tail currents in symmetrical K⁺) yielded a half-activation voltage of \(-21\) mV. Guillemaire et al. (1992) cloned a *rat cardiac Kv1.2* and expressed it in *Xenopus* oocytes, finding an activation threshold of \(-52\) or \(-57\) mV according to the amount of cRNA injected (low and high levels, respectively). Conductance curves from tail currents showed a half-activation voltage around \(-20\) mV in this case.

In order to have some data for comparison with the literature, I attempted to measure activation time constants for Kv1.2 clones. Despite signs of sigmoidal behaviour on the smaller depolarisations, some fairly consistent values emerged. Single activation \(\tau\)s fitted to rKv1.2 currents in CL1023 cells were 1-3 s at 0 mV, and 30-50 ms at +50 or +60 mV.

Activation \(\tau\)s fitted to mKv1.2 showed two components of about 120 and 980 ms at +2 mV, and 8 and 78 ms at +52 mV. Thus, the slow \(\tau\) values I obtained for mKv1.2 were similar in size to the single \(\tau\)s for rKv1.2. The fast \(\tau\)s in mKv1.2 resemble those reported by Paulmichl et al. (1991) for rKv1.2 (RAK clone) in oocytes: this (when stepped from \(-70\) mV) had
activation time constants ranging from 58 ms at -20 mV to 6 ms at +60 mV. For all the limitations of fitting exponential growth functions to a partially sigmoidal process, there is some consistency between Kv1.2 properties in oocytes and the MK2 line of CHO cells.

Deactivation
Deactivation kinetics were the point of maximum resemblance between Kv1.2 and the M-like current, so it was necessary to study Kv1.2 deactivation over the range of voltages conventionally used for M- and M-like currents. For rKv1.2, fitted fast τs were 60-300 ms, with a slow component in the range of seconds. For mKv1.2, fast τs were 10-40 ms, and slower τs around 100-300 ms. Disregarding the very slow component seen with rKv1.2, the 10-300 ms spread of Kv1.2 deactivation τs in the mammalian cell environment is similar in magnitude to M-current and M-like current values. Constanti & Brown (1981) reported a maximum deactivation τ of ~200 ms for the SCG M-current at 22°C. Robbins et al.’s (1992) value on the NG108-15 M-like current was 230 ms at 35°C.

Dendrotoxin block
My IC_{50} of 3.6 nM for dendrotoxin on rKv1.2 is close to literature values. Werkman et al. (1992) reported a dissociation constant of 2.8 nM for dendrotoxin on rKv1.2 in the same CL1023 cell line. Stühmer et al. (1989) obtained an IC_{50} of 4 nM on rKv1.2 (RCK5) in oocytes. Guillemare et al. (1992) found an IC_{50} of 2 nM in oocytes injected with low cRNA concentrations of a rat cardiac Kv1.2 clone. Apart from Guillemare et al.’s (1992) anomalous finding of reduced dendrotoxin block at high Kv1.2 expression levels, these values are very consistent. If the NG108-15 M-like current were based on Kv1.2, it ought to be sensitive to dendrotoxin.

I tested forskolin and dibutyryl cyclic AMP hoping to replicate the protein kinase A-mediated enhancement of the RAK clone reported by Huang et al. (1994). This PKA effect depends on an amino-terminal threonine residue, which appears to be conserved at position 46 in all mammalian Kv1.2 isoforms for which sequences are available (data from the Swiss Institute of Bioinformatics SWISS-PROT protein sequence database). In my experiments, these membrane-permeant activators of adenylate cyclase and protein kinase A failed to induce an increase in current, and forskolin consistently blocked the current in a dose-dependent fashion with an IC_{50} of 25 μM.
I tested lanthanum and zinc to compare Kv1.2 with the NG108-15 M-like current. For lanthanum on mKv1.2, my IC$_{50}$ was 25 μM, compared with 2.13 mM on the M-like current (Robbins et al. 1992). My IC$_{50}$ for zinc on mKv1.2 was 0.43 mM, compared with 0.011 mM on the M-like current (Robbins et al. 1992). These figures are sufficiently different to argue against Kv1.2 being a major constituent of the M-like current.

**Enhancement by repeated activations, temperature and niflumic acid**

The enhancement of Kv1.2 currents by repeated activations was plausibly attributed by Grissmer et al. (1994) to the existence of successive closed states of the channel. Thus, the channels open slowly on the first depolarisation following a resting period. After deactivation, the channels are initially in a closed state capable of rapid opening, but convert over seconds into a “resting” state. I confirmed this activation behaviour, and found additionally that deactivation tails were consistent whether activation during the preceding step was fast or slow. This suggests that the channels arrived at a uniform open state during the preceding step. The phenomenon illustrated in Figure 3.5, where an apparent inactivation is reversed during an ensuing gradual hyperpolarisation, might indicate either closure of some channels or a voltage-dependent substate of the open channels. Thus, these simple protocols provide evidence for at least two closed states of the channel, and probably one major open state (except perhaps at extreme positive potentials). The presence of inactivation complicates any attempt to devise a gating scheme for Kv1.2, and single-channel recordings would be needed to establish any further refinements.

My finding of a leftward shift in the Kv1.2 conductance/voltage curve and increased inactivation at raised temperatures accord with results for canine colonic smooth muscle Kv1.2. These latter currents, recorded at 32-35°C (an unusual condition in *Xenopus* oocytes), show a leftward shift in the conductance/voltage curve and a big increase in inactivation, compared with data obtained at room temperature (Russell et al. 1994).

A minimal explanation for the effects of repeated activation, raised temperature and niflumic acid is that all these conditions cause molecular adjustments pushing the channel towards the fast-opening closed state hypothesised above. Notably, the niflumic acid effect reversed rapidly on washout. As this only usually happened with drugs incapable of crossing the plasma membrane, I suggest that niflumic acid exerts its effect from an external binding site on the channel protein.
Niflumic acid is reported to increase some other potassium channel currents. 10 μM niflumic acid increased the xKCNQ1/minK current produced by injecting minK RNA into *Xenopus* oocytes, while 100 μM had a blocking effect (Busch et al. 1994a). The enhancement of KCNQ1/minK currents in oocytes by two related chloride channel blockers, mfenamic acid and DIDS, was mediated by the minK subunit rather than KCNQ1 (Busch et al. 1997a). 1-500 μM niflumic acid increased BK-type calcium-activated potassium currents through cloned Slo channels (Gribkoff et al. 1996). 500 μM niflumic acid activated KCNQ5 by inducing a -20 mV shift in voltage-dependence (Schroeder et al. 2000). Therefore, "activation" by niflumic acid is also found among other potassium channels of the voltage-gated superfamily.

**Kv1.2 as an M-like current constituent?**

Like Selyanko et al. (1995), but in contrast to Huang et al. (1993), I did not see any appreciable Kv1.2 current in NG108-15 cells. It is possible that NG108-15 cells used by different laboratories may develop different properties in culture. In fact, the NG108-15 traces shown by Huang et al. (1993) resemble the very square currents in Shahidullah et al. (1995a, b), apparently produced by a Kv1.2/Kv3.1 heteromultimer. Zhou et al. (1998) generated a Kv1.2 blocking antibody which inhibited this NG108-15 current, supporting the presence of Kv1.2 in the NG108-15 line studied by Peralta's group. They did not report whether the equivalent anti-Kv3.1 antibody had any effect on the NG108-15 current.

Kv1.2 deactivations occur on a time-scale similar to those of M- and M-like currents. However, M-currents and M-like currents have been described as non-inactivating over a voltage range from -20 mV to -60 mV. It is true that Kv1.2 currents (activated from rest) do not show strong inactivation over a similar voltage range of about 40 mV above the activation threshold. It is possible to lower the Kv1.2 current activation threshold in fibroblast and CHO cells to about -50 mV, using raised temperatures, niflumic acid treatment or pre-activation. However, this causes extra inactivation to set in below 0 mV. Kv1.2 is an unlikely candidate for an M-like current constituent unless its eccentric acceleration and inactivation behaviours can be shown to be an artefact of the expression systems used.

There is virtually no correspondence in the effects of blockers on Kv1.2 and on the M-like current. The insensitivity to dendrotoxin of the NG108-15 M-like current argues strongly against a contribution from Kv1.2 channels. This has also been commented on by Selyanko et al. (1995). There are two minor cautions regarding the use of dendrotoxin for identifying native Kv1.2 currents. One is the anomalous finding of Guillemare et al. (1992) that Kv1.2, when expressed at a high cRNA level in *Xenopus* oocytes, lost its tendency to inactivate and
its sensitivity to dendrotoxin. However, this has not been duplicated in other cell environments, and my mammalian cells expressed substantial Kv1.2 currents with no loss of dendrotoxin sensitivity. The other proviso relates to heteromultimers. For Kv1.1 at least, four channel subunits must contain the appropriate dendrotoxin-binding residues to allow high-affinity binding (Tytgat et al. 1995). However, even if a Kv1.2 heteromultimer with low dendrotoxin sensitivity were implicated in the NG108-15 M-like current, the most likely partner in the heteromultimer would be another Kv1 subunit, and one would expect this to have shown up during the original cloning of NGK1.

In summary, there are several lines of evidence that Kv1.2 is unlikely to contribute to the NG108-15 M-like current. The only kinetic data which are comparable between Kv1.2 in non-neuronal mammalian cells and the M-like current in NG108-15 cells are the rates of deactivation. The pharmacology of Kv1.2 and the M-like current is markedly different in respect of the blockers dendrotoxin, zinc, and lanthanum. In the case of linopirdine, there is a spread of sensitivities with the IC50 for the M-like current (24.7 μM: Noda et al. 1998) falling between that for the sympathetic M-current (3.4 μM: Lamas et al. 1997) and that for Kv1.2 (about 50 μM: D.G. Owen, personal communication). Bearing in mind the low selectivity of linopirdine (Lamas et al. 1997), this is insufficient basis for postulating a role of Kv1.2 in the M-like current. My provisional conclusion at the end of this work was that Kv1.2 was unlikely to form even part of the M-like current in the NG108-15 cell line I was using.
Chapter 4: mErg1a and the slow NG108-15 M-like current

4.1: Introduction: ERG channels

*Ether-à-go-go* (*eag*) potassium channels, like *Shaker*, were first detected from a mutation in the fruit fly *Drosophila*. *Eag* mutations cause leg shaking when the fly is anaesthetised with ether, resulting from hyperactivity of motor neurones (Schwartz & Bauer 1999). Warmke & Ganetzky (1994) identified a series of homologous genes, including *h-erg* (human *eag*-related gene) from human hippocampus. ERG currents, however, were first identified in the heart.

The human electrocardiogram features a series of waves designated P to T, reflecting the passage of depolarising and repolarising currents across the heart muscle. The landmarks of the cardiac action potential are designated P,Q,R,S and T waves. Particular abnormalities of the ECG are often diagnostic of clinical disorders (Schauf et al. 1990, p.333). The QRS complex, caused by depolarisation spreading rapidly across the ventricles, and the longer-lasting T wave reflecting ventricular repolarisation, give the name of the cardiac disorder long QT syndrome (LQTS). This may be inherited, but can also arise from treatment with certain antiarrhythmic and other drugs. It is identified by a lengthening of the Q-T interval, reflecting abnormal repolarisation of the ventricles. It is frequently accompanied by “torsade de pointes”, a distortion of the QRS axis, which may be a forerunner of ventricular fibrillation, causing sudden death in apparently healthy individuals. Curran et al. (1995) identified one of the genetic loci linked to LQTS as the newly-discovered potassium channel gene HERG, whose mRNA is strongly expressed in the heart.

Many blockers of HERG and its analogues have been discovered in the course of clinical drug development and monitoring. Class III antiarrhythmic drugs, which act by prolonging cardiac action potential duration and effective refractory period (Wang et al. 1999), include several known to induce LQTS. The benzenesulphonamide compound E-4031 selectively blocks the fast (I<sub>Kr</sub>) portion of the “delayed rectifier” currents which are found in cardiac myocytes and were later attributed to analogues of HERG. I<sub>Kr</sub> has the same bell-shaped current/voltage relationship (Sanguinetti & Jurkiewicz 1990) as reported for HERG.

WAY-123,398 is a 4-[(methylsulphonyl)amino]benzenesulphonamide having a 2-aminobenzimidazole substituent, synthesised by Wyeth-Ayerst Research as part of a programme to create improved Class III antiarrhythmics (Ellingboe et al. 1992). WAY-123,398 also acts on the “delayed rectifier” (Spinelli et al. 1993). Azimilide (NE-10064),
initially reported as a blocker of the "delayed rectifier", $I_{Ks}$ (Busch et al. 1994b), also inhibits $I_{Kr}$ and cloned HERG channels with an $IC_{50}$ in the micromolar range (Busch et al. 1998). The susceptibility of HERG to a wide range of drugs is reviewed in Taglialatela et al. (1998). From the natural world, Gurrola et al. (1999) identified a scorpion toxin selective for ERG currents (although the unpredictability of human LOTS suggests that cardiac ERG block would not contribute much to the incapacitating effect of scorpion venoms).

HERG (expressed in *Xenopus* oocytes) has a bell-shaped current/voltage relationship showing a peak at 0 mV, and undergoes block by lanthanum (Sanguinetti et al. 1995), caesium, barium and E-4031 (Trudeau et al. 1995). A short period of hyperpolarisation relieves inactivation of the channels, so any sudden return to depolarised potentials causes a large transient HERG current to appear. This mimics the situation in heart muscle undergoing premature depolarisation, where the HERG current serves to suppress the incipient arrhythmia (Miller 1996). HERG currents expressed in oocytes are similar to currents in cardiac myocytes. Single-channel properties closely resemble the native ones (Zou et al. 1997). HERG complexes *in vitro* with a new accessory subunit, MiRP1 (MinK-related peptide 1), in preference to minK, to give native-type currents (Abbott et al. 1999).

**HERG homologues and accessory subunits**

In the fruit-fly *Drosophila*, the channel homologous to HERG is the product of a gene at a locus previously named for the *seizure* mutant. The *seizure* mutant flies show spontaneous flight activity at high temperatures, paralleled by increased spontaneous firing in motor neurones when studied electrophysiologically. The *Drosophila* channel is therefore likely to participate in action potential repolarisation, and/or in setting a resting potential (Titus et al. 1997; Wang et al. 1997b).

HERG homologues (erg1) are prominently expressed in the hearts of several mammalian species besides man, in accord with their role as the $I_{Kr}$ constituent of ventricular potassium currents (Wymore et al. 1997). The mouse heart contains mRNA coding for two principal isoforms of the protein (Lees-Miller et al. 1997). The protein isoforms, mErg1a and mErg1b, coassemble in *Xenopus* oocytes to duplicate the kinetics of the native $I_{Kr}$ (London et al. 1997), though other researchers find no evidence for ERG1b protein (as opposed to mRNA) in adult rat, mouse or human heart (Pond et al. 2000). The mErg1a cDNA cloned by London et al. (1997) is derived from 15 exons of the mErg gene, and has an open reading frame of 1162 amino acids. The amino acid sequence is 96% similar to that of HERG. At an average 119.4 daltons per amino acid (Creighton 1993, p.4), the mErg1a channel subunit can be
expected to have a molecular weight of some 139 kD, and a tetrameric channel 555 kD, plus extra mass accounted for by glycosylation.

All three known members of the erg family are expressed in the rat nervous system. Rat erg3, present in the SCG as well as in the coeliac and superior mesenteric ganglia, displays a strongly-inactivating current with a negative threshold when expressed in oocytes (Shi et al. 1997).

ERG channels and the M-like current

The rationale for testing a Class III antiarrhythmic drug on the NG108-15 M-like current in September 1997 requires a little explanation. Similarities had been noted between the current complement in NG108-15 cells and that in certain pituitary tumour-derived cells. Hu & Shi (1997), studying NG108-15 cells, found no M-current, but did detect an inwardly-rectifying current with similar partial block by TEA, cobalt and cadmium. The GH3 line of rat pituitary adenoma cells expresses a potassium current, modulated by thyrotropin-releasing hormone, which is blocked partially by 5 mM caesium and totally by 10 mM TEA. Bauer et al. (1990) described it as inwardly-rectifying; Sankaranarayanan & Simasko (1996) as M-like.

Thus, it appeared possible that a current with particular pharmacological characteristics could manifest as either an M-like current or an inward rectifier, according to recording conditions and in the hands of different researchers. A possible channel identity for one or both of these currents emerged when Weinsberg et al. (1997) reported that the “inward rectifier” in the GH3/B6 subclone, recorded in high external potassium, was inhibited by the HERG-blocking drugs E-4031 and WAY-123,398. The GH3 “inward rectifier” current has recently been confirmed as rat ERG (r-erg1), which has 96% amino acid identity to HERG (Bauer et al. 1998).

Since it appeared that the M-like current in GH3 and NG108-15 cells might be an alternative form of the “inward rectifier”, it seemed appropriate to test the M-like current found in our laboratory’s NG108-15 cells with the “inward rectifier” blocker WAY-123,398. On the rat SCG M-current, WAY-123,398 had no effect. However, I saw a substantial inhibition of the NG108-15 M-like current at -20 mV. The inhibition was not total: closer observation revealed that the residual fraction of the M-like current had fast relaxations like the sympathetic M-current, while the inhibited fraction had slow relaxation kinetics. I therefore refer to these pharmacologically and kinetically distinct currents as the NG108-15 fast current and the NG108-15 slow current, respectively, or as the fast and slow portions, fractions, or
constituents. I have tried to avoid referring to them as "components" to avoid confusion with the two kinetic components of deactivation measured on the slow current.

Therefore, I and other members of the laboratory sought further evidence for an ERG current in NG108-15 cells. Polymerase chain reaction results obtained by collaborators in the Wellcome Laboratory for Molecular Pharmacology indicated that a message was present in NG108-15 cells for the mouse homologue of HERG. Experiments with the two known isoforms of mErg1 later narrowed down the likely identity of the NG108-15 slow current to the longer isoform, mErg1a (Selyanko et al. 1999). In collaboration with A.A. Selyanko, I compared the mErg1a current kinetically and pharmacologically with the slow portion of the NG108-15 M-like current, as described below. In addition to my own data, I cite some joint findings published in Selyanko et al. (1999).
4.2: Results

Heterologously-expressed mErg1a and the NG108-15 slow M-like current

I expressed the mErg1a channel in CHO hm1 cells (see Methods) by transfection with LipofectAmine Plus. The choice of this cell type was a result of (i) success with the MK2 line (also a CHO cell type) and (ii) the availability of cells already stably expressing muscarinic receptors.

The mErg1a currents were recorded 1-3 days after transfection, using the perforated-patch recording configuration. Functional channels were sparsely expressed during the whole of this period, with only about one cell in every nine having a current big enough to give reliable data. NG108-15 currents were normally recorded 7-14 days after plating, which regularly gave a standing M-like current of about 0.5-1.5 nA at the nominal holding potential of -20 mV. I have left the junction potential (-7.3 mV) uncorrected for consistency with the published data.

Kinetics

Activating mErg1a currents from rest is not an optimal way to study this current. Depolarisations from below the activation threshold gives current families (see Figure 5 in Selyanko et al. 1999) with untidy crossovers, as inactivation becomes increasingly dominant at more positive potentials. A version of the traditional M-current deactivation protocol was more informative, and was used for both NG108-15 slow currents and transfected mErg1a currents. For NG108-15, the holding potential was nominally -20 mV to give a maximal M-like current amplitude without activating other currents. With mErg1a, a holding potential of 0 mV was necessary to give a sufficiently large and stable standing current. A series of hyperpolarising voltage steps, at -10 mV increments, was given. The steps were 6 seconds in duration, to include most of the slow deactivation, and an interval of at least 40 seconds was normally allowed between pulses to allow for current recovery.

Inactivation and de-inactivation

With mErg1a, this long-step variant of the M-current protocol demonstrates the de-inactivation phenomenon seen also in HERG. Figure 4.1 shows mErg1a current families from two different cells. It should be remembered that these are responses to hyperpolarising voltage steps, and that mErg1a owes its paradoxical kinetic behaviour to very fast voltage-dependent inactivation and de-inactivation.
Thus, moderate hyperpolarising steps from 0 mV cause a transient increase in the current. This is because a large number of available channels have been inactivated at 0 mV. On stepping to a more negative potential where the voltage-dependent inactivation is lower, many of these channels lose inactivation almost instantaneously and the current rises to a peak. The ensuing decline in current (while resembling the inactivation of an A-current) is caused by voltage-dependent deactivation.

The extent of steady-state inactivation at 0 mV varies in CHO cells, illustrated by the difference between the two cells in Figure 4.1. In the upper record, steady-state inactivation is high, and a hyperpolarising step to −50 mV initially releases considerably more current than was visible at 0 mV. The slow deactivation means that the current amplitude at the end of a 6-second hyperpolarisation to −30 mV is still greater than the holding current at 0 mV. With larger hyperpolarisations, to −40 or −50 mV, the transient peak level of the current is still a long way above the holding current, but the deactivation process is becoming rapid enough to reduce the current at the end of the step to below the holding current.

The lower record in Figure 4.1 is an example of a mErg1a current with comparatively low steady-state inactivation, similar to that seen on the NG108-15 slow current. In this case, only the −10 mV step gives a current exceeding the holding current after 6 seconds, and the amplitude of the deactivation tail elicited by the −50 mV step never exceeds the holding current. This current family also shows, at the beginning of the step back to 0 mV following the hyperpolarising step, a sudden drop in current that probably represents the inactivation process induced by depolarisation, after which the current slowly increases back towards the holding level.

Thus, this protocol largely separates inactivation, deactivation and activation. Inactivation can be observed retrospectively from the jump in the current on hyperpolarising, while deactivation accounts for the time-dependent decline during the voltage step. The onset of inactivation on returning to a depolarised potential is so rapid that it barely contaminates the slow activation process.
Figure 4.1: Two examples of mErg1a in CHO cells

Current families in two different CHO cells evoked by a protocol (top) of hyperpolarising steps, 6 seconds in duration, from a 0 mV holding potential. On the upper record, currents are increased even at the ends of the first two steps, to −10 and −20 mV. At −30 mV and more negative potentials, the current deactivates from an initial peak which is much higher than the holding current at 0 mV.

Inactivation was variable; the lower record shows a mErg1a current family with comparatively low steady-state inactivation at 0 mV.

Whatever the extent of inactivation, the deactivation at −50 mV (picked out in grey) reliably had a large amplitude: this was the potential chosen for drug tests.
Figure 4-1
The presence of a mErg1a-like current in NG108-15 cells is demonstrated in the following Figures: Figure 4.2, 4.3 and 4.4. Figure 4.2 (upper record) shows a representative NG108-15 M-like current, recorded in response to 6-second steps from −20 mV to progressively more negative potentials, by −10 mV increments. I have fitted the current traces (grey) with a three-exponential fit (dotted black lines), which are extrapolated beyond the beginning and end of the deactivation steps. Table 1 lists the time constants. These fit lines demonstrate that most but not all of the deactivation occurs within 6 seconds on steps to −30 to −60 mV; the slowest τs fitted being of the order of seconds. Thus, the typical NG108-15 M-like current contains an element which deactivates very slowly indeed. A medium-length component has a τ of several hundred milliseconds, and the fastest is around 100 ms for all but the most negative voltages. The centre record of Figure 4.2 shows the currents in the same cell after applying 10 μM WAY-123,398. The current deactivations here are fitted with a single exponential equation, which describes a current which settles rapidly on hyperpolarising, with time constants of around 100 ms (Table 1), as found for the fastest of three components fitted to the whole current. The traces in the bottom panel of Figure 4.2 are obtained by digital subtraction of the current in WAY-123,398 from the whole current. This current constituent has time constants (Table 1) of several hundred milliseconds and several seconds, corresponding to the two slower components present in the whole current.

Figure 4.3 compares the mErg1a current from the bottom of Figure 4.1 and the WAY-123,398-sensitive subtraction current from Figure 4.2. While the mErg1a current deactivations are from a holding potential of 0 mV and the NG108-15 WAY-123,398-sensitive current is from −20 mV, there is a close resemblance. Both currents relax on a similar time-scale, and the smaller hyperpolarisations cause a transient increase in current before the deactivation process is well under way.

Figure 4.4 demonstrates the selectivity of WAY-123,398 for mErg1a and the NG108-15 slow current, compared with the other currents I have studied in this work. Thus, 10 μM WAY-123,398 blocks much of the mErg1a standing current and only a minimal deactivation tail remains. On the whole NG108-15 current (recorded in this case from a holding potential of 0 mV), the standing current is appreciably decreased and only a small, fast deactivation is seen. In both cases an intermediate concentration of WAY-123,398, 1 μM, gave a partial inhibition of the slow tail-current. By contrast, the KCNQ2+KCNQ3 current in CHO hm1 cells and the mKv1.2 current in MK2 CHO cells are not inhibited at all by 10μM WAY-123,398. The same result was obtained on a total of three cells for both KCNQ2+KCNQ3 and mKv1.2.
Figure 4.2 therefore indicates one way in which the NG108-15 slow current, otherwise masked both by the fast current and by the capacity transients of these large and non-spherical cells, can be singled out. 10 μM of WAY-123,398 gave about 95% block of both currents (see later Figures 4.10 and 4.11) and 20 μM gave no additional block. It was therefore considered that the current blocked by 10 μM WAY-123,398 constituted the mErg1a-like component of the NG108-15 current, while the residual fast current in 10 μM WAY-123,398 had negligible contamination by the slow current. Digital subtraction of the current in 10 μM WAY-123,398 from the pre-drug control makes it possible to visualise the slow current in isolation, freed even from the usual transients. (Another method is by selection of exponential fit components: see later).

Such a direct comparison, of a difference current with a cloned channel recorded in the absence of a blocker, is subject to some limitations. WAY-123,398 block of the mErg1a-like current does not appear to be strongly voltage-, state- or time-dependent. However, with a deactivation protocol as used here, and a mErg1a-like current component, several changes might appear in the profile of the difference current according to the behaviour of the blocker. A voltage-dependent blocker with a stronger effect at more positive voltages could raise the apparent voltage threshold: block might be strong at −20 mV, moderate at −30 mV, and weak at −50 mV, giving a negligible difference current at −50 mV. A state-dependent blocker could favour open, closed or inactivated channels. Selective block of open channels would suppress the standing current, while still allowing inactivated channels to be released by de-inactivation. The difference current after subtraction would show a dominant standing current and less evidence of the large Erg de-inactivation tails, and look more like a delayed rectifier. A preference of the blocker for inactivated channels would cause little or no decline in standing current (depending on the equilibrium between active and inactivated channels at the holding potential), but tails would be smaller in the drug-treated current, therefore larger in the difference current, exaggerating the normal Erg pattern. If the onset or removal of block is time-dependent over the same time-scale as the deactivation process, this could add a further distortion of the current behaviour by accelerating or slowing the drug-treated tail currents. The corresponding difference currents would tend to show, respectively, a rise followed by a downward creep, or a fall followed by an upward creep – quite unlike the normal profile of the current.

In such a case, where block by a channel-specific drug gave rise to a difference current whose kinetics did not match those of the cloned channel, it would be necessary to compare the cloned channel current and the native current component under identical conditions. Recording the cloned channel current with and without the blocker, and using the same
subtraction method as for the native current, should duplicate the difference current if the underlying channels are the same.

In the NG108-15 slow current, the initial increase in amplitude on hyperpolarising is smaller than for mErg1a in CHO cells. This can probably be explained by the less depolarised holding potential used on NG108-15, which produces lower inactivation (although a small state-dependence of WAY-123,398 block might contribute). Also as in mErg1a-transfected CHO cells, I found some variation in the amount of inactivation removed by hyperpolarisation in NG108-15 slow currents: among 19 cells, the transient increase in current on modest hyperpolarisations was usually small but visible. In one case it was non-existent, and one cell had peak currents several times the standing current amplitude. The current I have used as an example in Figure 4.2 and Figure 4.3 has slightly more steady-state inactivation than average for these 19 cells. (Note also the error-bars on the mean normalised peak amplitudes in Figure 4.6, which indicate the variation in the proportion of current released by de-inactivation).
Figure 4.2: Separation of NG108-15 M-like current constituents

(Top) The standard protocol used on NG108-15 cells was 6s long, as for mErg1a, but the holding potential was -20 mV.

(Upper record) A specimen of a mixed M-like current, with a three-exponential fit superimposed.

(Centre record) The same cell, during perfusion with 10 μM of the HERG blocker WAY-123,398, has lost the slow components of the deactivations; the superimposed single-exponential fit is adequate.

(Bottom record) Difference current, obtained by digital subtraction of the centre record from the upper record, with superimposed two-exponential fit. This current shows typical ERG-type de-inactivation during the hyperpolarising steps.
Whole NG108-15 M-like current, fitted with three exponential components

M-like current in 10 μM WAY-123,398, fitted with one exponential component

WAY-123,398-inhibited current by subtraction, fitted with two exponential components
Table 1: Deactivation time constants for the fitted curves shown in Figure 4.2

1. Whole current.

<table>
<thead>
<tr>
<th>Membrane potential (mV)</th>
<th>Fast $\tau$ (ms)</th>
<th>Medium $\tau$ (ms)</th>
<th>Slow $\tau$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-30</td>
<td>143.9</td>
<td>707.9</td>
<td>4817.9</td>
</tr>
<tr>
<td>-40</td>
<td>115.1</td>
<td>796.2</td>
<td>3527.0</td>
</tr>
<tr>
<td>-50</td>
<td>111.1</td>
<td>673.6</td>
<td>3061.0</td>
</tr>
<tr>
<td>-60</td>
<td>121.8</td>
<td>610.7</td>
<td>2810.2</td>
</tr>
<tr>
<td>-70</td>
<td>98.5</td>
<td>451.4</td>
<td>1502.2</td>
</tr>
<tr>
<td>-80</td>
<td>83.5</td>
<td>211.2</td>
<td>845.5</td>
</tr>
<tr>
<td>-90</td>
<td>18.4</td>
<td>55.4</td>
<td>697.1</td>
</tr>
<tr>
<td>-100</td>
<td>30.8</td>
<td>64.8</td>
<td>129.4</td>
</tr>
</tbody>
</table>

2. Fast current (in the presence of 10 $\mu$M WAY-123,398).

<table>
<thead>
<tr>
<th>Membrane potential (mV)</th>
<th>$\tau$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-30</td>
<td>172.0</td>
</tr>
<tr>
<td>-40</td>
<td>152.5</td>
</tr>
<tr>
<td>-50</td>
<td>117.7</td>
</tr>
<tr>
<td>-60</td>
<td>106.5</td>
</tr>
<tr>
<td>-70</td>
<td>105.4</td>
</tr>
<tr>
<td>-80</td>
<td>39.8</td>
</tr>
<tr>
<td>-90</td>
<td>46.4</td>
</tr>
<tr>
<td>-100</td>
<td>41.0</td>
</tr>
</tbody>
</table>

3. Slow current (by subtraction of WAY-123,398-inhibited current from whole current).

<table>
<thead>
<tr>
<th>Membrane potential (mV)</th>
<th>faster $\tau$ (ms)</th>
<th>slower $\tau$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-30</td>
<td>869.5</td>
<td>4827.5</td>
</tr>
<tr>
<td>-40</td>
<td>974.0</td>
<td>4005.7</td>
</tr>
<tr>
<td>-50</td>
<td>849.9</td>
<td>4204.7</td>
</tr>
<tr>
<td>-60</td>
<td>359.0</td>
<td>1617.7</td>
</tr>
<tr>
<td>-70</td>
<td>191.1</td>
<td>762.1</td>
</tr>
<tr>
<td>-80</td>
<td>161.2</td>
<td>728.0</td>
</tr>
<tr>
<td>-90</td>
<td>110.3</td>
<td>791.8</td>
</tr>
<tr>
<td>-100</td>
<td>59.0</td>
<td>147.9</td>
</tr>
</tbody>
</table>
**Figure 4.3: a direct comparison of mErg1a and the NG108-15 slow current**

(Above) Voltage protocol (holding at 0 mV) and mErg1a current deactivating from 0 mV.

(Below) Voltage protocol (holding at −20 mV) and WAY-123,398-sensitive current in NG108-15.
Figure 4.3

WAY-123,398-sensitive current in NG108-15

mErg1a in CHO hm1
Figure 4.4: Selectivity and concentration-dependence of WAY-123,398 block
The effect of WAY-123,398 on four currents, with the relevant voltage protocol shown above each one.

(Above left) mErg1a. Reduction of relaxation at −50 mV by 1 μM WAY-123,398. Substantial decrease in the holding current at 0 mV, and virtual elimination of the relaxation at −50 mV, by 10 μM WAY-123,398.

(Above right) M-like current in NG108-15. Reduction in holding current at 0 mV, and reduction in the −50 mV relaxation, by 1 μM WAY-123,398. Further reductions in both by 10 μM WAY-123,398, with the residual relaxation being faster.

(Below left) KCNQ2+KCNQ3 current expressed in a CHO hm1 cell. No effect of 10 μM WAY-123,398. Same result in a further 2 cells.

(Below right) mKvl.2 in an MK2 cell. No effect of 10 μM WAY-123,398. Same result in a further 2 cells.
Figure 4-4
**Current/voltage relationships: deactivation and its measurement**

Figure 4.5 clarifies the fitting procedure used for characterising mErgla and NG108-15 slow currents, using a mErgla current as an illustration. "End-of-step" values represent the mean current amplitude over a short time interval near the end of the voltage step, leak-subtracted either before analysis in Clampfit or retrospectively in Excel (see Chapter 2, Methods: Leak-subtraction of data records, p.34). "Instantaneous" or "peak" current values for both mErgla and the NG108-15 slow current, at the start of hyperpolarising voltage steps, are standardised by back-fitting or calculation (see Chapter 2, Methods: Fitting deactivation tails, p.34). For mErgla expressed in CHO cells, and WAY-123,398-sensitive subtraction currents in NG108-15, deactivation manifests as a bi-exponential decline of the current during the 6-second hyperpolarising step. Thus, the peak amplitude of the deactivation tail is obtained by back-extrapolating a two-exponential fit to the first sample of the hyperpolarising voltage step, and leak-subtracting as appropriate.

Figure 4.6 plots the combined "instantaneous" and end-of-step I/V relationships for mErgla in CHO hml cells, and for the WAY-123,398-sensitive NG108-15 slow current as obtained by subtraction. When leak-subtracted mErgla current amplitudes are normalised, with the standing current at 0 mV defined as 1, the maximum averaged "instantaneous" current (at -40 mV) is over 3. Thus, considering that there is a drop in driving force on hyperpolarising, more than 70% of the available channels are inactivated when holding at 0 mV. In the case of the NG108-15 slow current, when the standing current at -20 mV is set at 1, the maximum normalised "instantaneous" current (at -50 mV) is 1.7. Close inspection of Figure 4.6 also reveals that there is a small residual NG108-15 current even at -60 mV, whereas mErgla is barely active at -50 mV.

**Reversal potential**

The solutions used for NG108-15 and mErgla (and for KCNQ – see Chapter 5) channel recordings included 110 mM potassium internally and 2.5 mM potassium externally. This gives a theoretical reversal potential of -95.54 mV, according to the equation $V_{rev} = (\ln([K']_o/[K']_i))RT/ZF$. My actual values, obtained from the point where "instantaneous" and end-of-step I/V curves cross, were nearer -80 mV: -78 mV for mErgla-transfected CHO hml cells and -84 mV for the WAY-123,398 subtraction current in NG108-15 cells. The discrepancy can be partially accounted for by the uncorrected liquid junction potential of -7.3 mV (see Chapter 2, Methods). It is possible that slow clearance of extracellular potassium build-up around the cell membrane may also pull the measured reversal potential in a positive direction. However, the holding potentials of 0 and -20 mV used for these
experiments produced stable standing currents; if the holding current drifted, I perfused with fresh bath solution until it was stable before continuing recording. Any effect of raised extracellular potassium was therefore rapid, and applied consistently to all current measurements.

I have used unmanipulated current/voltage data to characterise mErg1a; conductance calculation would require a specialised protocol to avoid inactivation effects. Therefore, these reversal potentials serve mainly as evidence for the potassium selectivity of the channels.

**Deactivation time constants**

In addition to the current amplitude measurements used to construct the I/V curves, the above fitting procedure also provides another basis for comparing the two currents: deactivation time constants. These are shown in Figure 4.7. The upper graphs demonstrate that the two fitted $\tau$s are similar in magnitude for mErg1a (deactivating from 0 mV) and the NG108-15 slow current (deactivating from $-20$ mV). Both fast and slow $\tau$ values decrease with increasing hyperpolarisation, as relaxations become faster. Further, the amplitudes corresponding to the two $\tau$s show a similar relationship to voltage in both currents (centre graphs). The slower component contributes more of the relaxation on the smaller hyperpolarisations, and the faster component becomes predominant negative to $-40$ or $-50$ mV. When each amplitude is plotted as a proportion of the total relaxation amplitude (lower graphs), the contribution of the two components is seen to be strongly voltage-dependent.

Most of the deactivation $\tau$s obtained for mErg1a and NG108-15 currents occupy less than half of the 6-second step duration, allowing some confidence in the values obtained (see Methods). A two-exponential fit of WAY-123,398-sensitive slow M-like currents (19 cells) gave time constants with ranges 50-900 ms and 1800-4000 ms. Equivalent values for CHO hm1 cells expressing mErg1a (23 cells) were 50-400 ms and about 500-4000 ms.
Figure 4.5: Fitting methods for mErg1a and the NG108-15 slow current
How I obtained end-of-step and “instantaneous” current/voltage relationships, using a mErg1a current as an example.
END-OF-STEP I/V

...extrapolate back to an extra cursor here, at the start of deactivation, or calculate amplitudes in spreadsheet program.

The back-fitted current amplitude corresponds to the theoretical current level at the first sample of the deactivation step. Leak-subtract if necessary, and normalise to the holding current (from end-of-step I/V).

INSTANTANEOUS I/V

place fitting cursors about here, and fit bi-exponential curves to tail-currents...

...either correct for the leak in Clampfit and re-measure the I/V, or construct a leak line in the analysis spreadsheet and subtract leak values from the current amplitudes.

Normalise end-of-step amplitudes against the holding current.

Take the holding current amplitude and the leak line from the end-of-step I/V to use in the peak I/V.

End-of-step current: measure average value between two cursors placed around here...

...choose a linear sub-threshold region of the I/V and extrapolate a leak line...

...extrapolate back to an extra cursor here, at the start of deactivation, or calculate amplitudes in spreadsheet program.

The back-fitted current amplitude corresponds to the theoretical current level at the first sample of the deactivation step. Leak-subtract if necessary, and normalise to the holding current (from end-of-step I/V).
Figure 4.6: Voltage-sensitive de-inactivation of mErg1a and the NG108-15 slow current

(Top) Back-fitted peak (grey) and end-of-step (black) mErg1a amplitudes, ± s.e.m., for currents evoked by the hyperpolarising step protocol (Figure 4.1), normalised to the amplitude at 0 mV, averaged, and plotted against voltage. Peak currents are obtained back-fitting; also note that “end-of-step” currents are not steady-state currents, since the slow relaxations are not all completed within the 6-second step. A true steady-state current/voltage curve would probably show a less pronounced bell-shape.

(Bottom) Back-fitted peak (grey) and end-of-step (black) WAY-123,398-sensitive NG108-15 current amplitudes ± s.e.m., for deactivations in response to hyperpolarisations from −20 mV. Currents were obtained by digital subtraction of records in the absence and presence of the drug (as in Figure 4.2), normalised to the current amplitude at −20 mV, averaged, and plotted against voltage. As for mErg1a in CHO cells, a true steady-state current would show less of a bell-shape (the extrapolated fit lines in Figure 4.2 give an idea of the current amplitudes at >15 seconds).
WAY-123,398-sensitive component of the M-like current in 19 NG108-15 cells

Figure 4-6
Figure 4.7: mErg1a and NG108-15 slow current kinetics compared

(Top) Average time constants, ± s.e.m., from two-exponential fits to 23 mErg1a currents in CHO cells (left) and 19 WAY-123,398-sensitive currents in NG108-15 cells (right).

(Centre) Average amplitudes, mathematically corrected, ± s.e.m., corresponding to the two τs plotted above, for mErg1a (left) and WAY-123,398-sensitive NG108-15 currents (right).

(Bottom) Amplitudes for the two deactivation components in each cell were summed, and each component was expressed as a proportion of the total amplitude. Averaged values ± s.e.m., are shown for mErg1a (left) and the WAY-123,398-sensitive NG108-15 current (right).
Figure 4-7
Activation

I obtained a limited amount of activation data for mErg1a as a by-product of the deactivation protocol. The reactivation traces from the various potentials to 0 mV were fitted by two exponential components. Although the smaller hyperpolarising steps commonly cause an overall increase in current (since inactivation has been removed), returning to 0 mV causes the current to drop below the original 0 mV current. This is followed by a slow rise back to the holding current amplitude. A very rapid drop in current at the beginning of the step back to 0 mV is visible on the lower record in Figure 4.1, and apparently represents the inactivation process induced by depolarisation. This initial region of the step was excluded from the fit, and the usual back-fitting methodology was used. The resulting τs for activation from voltages between −110 and −10 mV to 0 mV are about 700-1200 ms and 4400-7000 ms, measured over a duration of 15s. I have plotted these data on a logarithmic scale in Figure 4.8, along with a log-scale version of the mErg1a deactivation τs. While the deactivation τs (lower graph) are voltage-dependent, this sample of activation data shows little effect of the initial voltage. This contrasts with the notable behaviour of the related channel r-eag, which activates more slowly from strongly negative potentials (Ludwig et al. 1994). Like deactivation, activation has two components. Unfortunately, the equivalent data for reactivations to −20 mV of the NG108-15 slow current, even when isolated on subtracted records, are too slow and noisy to fit with any confidence.
Figure 4.8: mErg1a activation and deactivation compared

(Top) $\tau \pm $ s.e.m. from a 2-exponential fit to activations from hyperpolarised potentials back to the holding potential of 0 mV, plotted on a logarithmic y-axis against the potential for the previous step.

(Bottom) $\tau \pm $ s.e.m. for mErg1a deactivation, as shown in Figure 4.7 (top left) but plotted on a logarithmic y-axis for comparison.
Figure 4-8
Step potential (mV)

Activation τ (ms)

Deactivation τ (ms)

Previous membrane potential (mV)

Step potential (mV)
NG108-15 current dissection

While a whole current constituent can be isolated by subtracting currents recorded in the absence and presence of a drug such as WAY-123,398, one cannot apply additional drugs to the resulting mathematical abstraction. In order to assess the effects of other drugs on the slow fraction of the NG108-15 M-like current, alongside heterologously-expressed mErg1a, another method was necessary. This, more universally applicable, method involved fitting three exponential components to the whole NG108-15 M-like current.

The top trace of Figure 4.2 shows an example of an entire M-like current fitted with three exponential components; the fit lines are extrapolated beyond the ends of the deactivating voltage step. The lower traces show the current remaining in WAY-123,398, fitted with one exponential, and the WAY-123,398-sensitive (presumed mErg1a) difference current, fitted with two exponential components.

The WAY-123,398-resistant current relaxed rapidly with time constants ~50-100 ms. These are similar to the values found for the fastest component of the whole M-like current, and for the classic M-current (see Chapter 5). The WAY-123,398-sensitive current and the two slower components of the three-exponential fit had time constants ~100-800 ms and ~1000-2000 ms. Summing the corrected amplitudes for these two slower components gave the amplitude of the proposed mErg1a current. Thus, it was possible to assess the proportional contribution of the two channel types to the M-like current.

Figure 4.9 compares the properties of the ~20 to ~50 mV relaxation for sixteen NG108-15 cells for which good separated fast and slow current records were available. These relaxations were fitted as described above: the original current with three exponential components, the WAY-123,398-resistant current with one exponential, and the WAY-123,398-sensitive current with two. The results for the separated currents were reassembled and compared with those for the original current. The time constants (upper graph) and amplitude contributions (lower graph) show an impressive resemblance, testifying that the fitting procedure was well-founded – and hence that WAY-123,398 is a selective and complete blocker of the slow current.

This sample of 16 cells was chosen for WAY-123,398 application because of visibly slow relaxations; the contribution of the slow current was about 72%. Similar fits to my data from the (unselected) total of 65 cells established a slow current contribution of 70%.
The channels whose closure makes up the relaxation are those which were open at –20 mV, plus any released from inactivation by the hyperpolarising step. Figure 4.6 shows that this inactivation is less pronounced in NG108-15 cells than for mErg1a in CHO cells under the conditions used. Thus, the slow current might be expected to comprise something under 70% of the standing current. I used current/voltage data to obtain a leak-corrected amplitude for the current at –20 mV, in the presence and absence of WAY-123,398. On 19 cells, the –20 mV current amplitude was reduced 66 ± 2% by 10 µM WAY-123,398.
Figure 4.9: Dissection and reconstitution of the M-like current

(Top) Average $\tau \pm$ s.e.m. from separate fits to WAY-123,398-resistant and WAY-123,398-sensitive currents deactivating at $-50$ mV in sixteen NG108-15 cells (above). Compare $\tau$s from a three-exponential fit to whole M-like current deactivations (below).

(Bottom) The contribution of the amplitude for each deactivation component was calculated ($\pm$ s.e.m.) as a proportion of the total, for M-like currents separated by WAY-123,398 (upper bar) and for whole M-like currents (lower bar).
Whole M-like current (3-exponential fit) + WAY-123,398-resistant (1-exponential fit) + WAY-123,398-sensitive (2-exponential fit)

Amplitudes

proportion of total current amplitude

WAY-123,398-resistant (1-exponential fit) + WAY-123,398-sensitive (2-exponential fit)

NG108-15 currents: fitted τs

<table>
<thead>
<tr>
<th>slow</th>
<th>mid</th>
<th>fast</th>
</tr>
</thead>
</table>

121
Pharmacology

The multi-exponential fit method was used to study the effect of several potassium channel blockers on the slow portion of the M-like current. Drugs were applied cumulatively in doses following a 1,3,10,30… pattern, giving intervals of about 0.5 log unit. The relaxation at -50 mV was assessed for a control trace, and after reaching a stable current amplitude at each dose of the blocker. For the NG108-15 current, three exponential components were back-fitted, and the mathematically-corrected amplitudes (see Methods) were summed for the two slower ones. For mErg1a, the total amplitude of the relaxation from 0 to -50 mV was measured. The inhibition of the current was then calculated, plotted against the concentration of drug, and fitted with the Hill equation (using Microcal Origin’s “logistic” equation; see Methods) to obtain a Hill slope and a half-maximal inhibiting concentration.

Figure 4.10 shows concentration/response curves for WAY-123,398, the antiarrhythmic drug azimilide, THA and tetraethylammonium on mErg1a currents expressed in CHO hm1 cells. Figure 4.11 shows the corresponding data for the NG108-15 slow current. Table 2 summarises my results. Agreement between mErg1a and the NG108-15 slow current is generally good. [There are some small discrepancies between the IC50s I give here and the figures published in Selyanko et al. (1999) for the same sets of data, probably attributable to choice of fitting criteria. My fits, as given here, are all constrained to a Ymax of 100%].

Fitting curves to the drug inhibition data for each individual cell, and using a two-tailed Student’s t-test (in Excel) for comparison between the mErg1a and NG108-15 currents, gave the P values shown in the lower part of Table 2. In no case was a P value of less than 0.05 (which would suggest a significant difference between data for mErg1a and the NG108-15 slow current) obtained. The outcome was similar when a cross-comparison was made between data for azimilide and TEA inhibitions of mErg1a and the NG108-15 fast current. However, see also the point in Chapter 6 (p.178), that the actions of several drugs considered as a group give a clearer indicator of channel identity than the individual potencies. Thus, the low linopirdine block of mErg1a and the slow NG108-15 current, and the lack of WAY-123,398 block of KCNQ channels and the fast NG108-15 current, where IC50S could not be obtained, form a persuasive part of the pattern.
Figure 4.10: Concentration/response curves for four blockers on the mErg1a current
All data are plotted as mean inhibition ± s.e.m.

(Top left) WAY-123,398 (n=3)
Hill equation fit parameters:
$Y_{\text{max}} = 100\%$
$IC_{50} = 0.29 \pm 0.01 \mu M$
$n_H = 0.88 \pm 0.02$

(Top right) Azimilide (n=4)
Hill equation fit parameters:
$Y_{\text{max}} = 100\%$
$IC_{50} = 5.84 \pm 0.28 \mu M$
$n_H = 0.85 \pm 0.03$

(Bottom left) THA (n=3)
Hill equation fit parameters:
$Y_{\text{max}} = 100\%$
$IC_{50} = 58.12 \pm 8.48 \mu M$
$n_H = 0.70 \pm 0.07$

(Bottom right) TEA (n=3)
Hill equation fit parameters:
$Y_{\text{max}} = 100\%$
$IC_{50} = 22.62 \pm 2.97 \text{ mM}$
$n_H = 0.55 \pm 0.04$
% inhibition of control mErg1a current

Figure 4-10

[Graph showing concentration of THA (µM) vs. % inhibition with error bars for (n=3)]

[Graph showing concentration of WAY-123,398 (µM) vs. % inhibition with error bars for (n=3)]

[Graph showing concentration of TEA (mM) vs. % inhibition with error bars for (n=3)]

[Graph showing concentration of Azimilide (µM) vs. % inhibition with error bars for (n=4)]
Figure 4.11: Concentration/response curves for four blockers on the NG108-15 slow current
All data are plotted as mean inhibition ± s.e.m.

(Top left) WAY-123,398 (n=3)
Hill equation fit parameters:
\( Y_{\text{max}} = 100\% \)
\( IC_{50} = 0.31 \pm 0.03 \mu\text{M} \)
\( n_H = 1.05 \pm 0.12 \)

(Top right) Azimilide (n=4)
Hill equation fit parameters:
\( Y_{\text{max}} = 100\% \)
\( IC_{50} = 8.39 \pm 0.47 \mu\text{M} \)
\( n_H = 0.96 \pm 0.05 \)

(Bottom left) THA (n=3)
Hill equation fit parameters:
\( Y_{\text{max}} = 100\% \)
\( IC_{50} = 23.54 \pm 2.72 \mu\text{M} \)
\( n_H = 0.91 \pm 0.09 \)

(Bottom right) TEA (n=3)
Hill equation fit parameters:
\( Y_{\text{max}} = 100\% \)
\( IC_{50} = 32.24 \pm 7.18 \text{mM} \)
\( n_H = 0.89 \pm 0.18 \)
Figure 4.11

% Inhibition of control NG108-15 slow current

THA
(n=3)

WAY-123,398
(n=3)

TEA
(n=3)

Azimilide
(n=4)
Table 2: IC\textsubscript{50} values for inhibition of mErg1a and NG108-15 slow current by various drugs

All IC\textsubscript{50} values are quoted ± s.e.m., firstly, as calculated on averaged inhibition data:

<table>
<thead>
<tr>
<th>DRUG</th>
<th>mErg1a in CHO hm1</th>
<th>NG108-15 slow</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAY-123,398 (µM)</td>
<td>0.29±0.01</td>
<td>0.31±0.03</td>
</tr>
<tr>
<td>azimilide  (µM)</td>
<td>5.84±0.28</td>
<td>8.39±0.47</td>
</tr>
<tr>
<td>THA         (µM)</td>
<td>58.1±8.5</td>
<td>23.5±2.7</td>
</tr>
<tr>
<td>TEA         (mM)</td>
<td>22.6±3.0</td>
<td>32.2±7.2</td>
</tr>
<tr>
<td>linopirdine (µM)</td>
<td>&gt;30</td>
<td>&gt;&gt;30</td>
</tr>
</tbody>
</table>

and secondly from fitting data individually for each cell, averaging and applying a t-test:

<table>
<thead>
<tr>
<th>DRUG</th>
<th>mErg1a in CHO hm1</th>
<th>NG108-15 slow</th>
<th>P value, mErg1a vs. NG108-15 slow</th>
<th>P value, mErg1a vs. NG108-15 fast</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAY-123,398 (µM)</td>
<td>0.40 ± 0.14</td>
<td>0.71 ± 0.50</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>azimilide  (µM)</td>
<td>6.88 ± 1.23</td>
<td>8.75 ± 3.10</td>
<td>0.59</td>
<td>0.13</td>
</tr>
<tr>
<td>THA         (µM)</td>
<td>63.33 ± 22.05</td>
<td>11.83 ± 1.88</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>TEA         (mM)</td>
<td>41.67 ± 29.3</td>
<td>14.25 ± 4.64</td>
<td>0.33</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Note: I obtained the results for WAY-123,398 early on in the programme of work, before a holding potential of −20 mV had been standardised for NG108-15 cells. Thus, for this blocker only, the NG108-15 currents were measured on relaxations at −50 mV from a holding potential of 0 mV rather than −20 mV. There is no reason to suppose that WAY-123,398 block is appreciably voltage-dependent, and the results are in this case recorded for identical voltage steps for CHO hm1 and NG108-15 cells.
Accommodation

I also studied the effect of blockers on NG108-15 firing. Figure 4.12 shows data obtained in current-clamp mode on two NG108-15 cells. I applied a series of incremental current steps, and selected cells showing consistent phasic firing for drug treatment. Several cells were discarded because of instability in the resting potential, or loss of firing over time. It is unfortunate that the recording instrumentation could not be easily switched to voltage-clamp to check the properties of the M-like current. Nevertheless, I consider that the two cells illustrated in Figure 4.12 display the normal "braking" action of the total M-like current and its susceptibility to blocking drugs at room temperature. What emerges is that eliminating the mErg1a constituent with 10 µM WAY-123,398 only slightly increases the spiking rate. In a cell treated with WAY-123,398 alone, firing stops at the same time-point as in the control record, indicating that the duration of firing is controlled by some independent factor. This factor is revealed by applying the KCNQ channel blocker linopirdine at an M-current-blocking dose of 30µM. Continuous spiking ensues, demonstrating that the major influence on NG108-15 spike accommodation resembles the sympathetic M-current in pharmacology.
Figure 4.12: Effects of KCNQ and ERG block on NG108-15 firing

(Left) Current-clamp protocol and voltage recordings from an NG108-15 cell to which the KCNQ channel-/M-current blocker linopirdine was applied, followed by WAY-123,398. Linopirdine induces continuous spiking; WAY-123,398 marginally increases the firing rate.

(Right) Protocol and voltage recordings made in current-clamp mode, on an NG108-15 cell in which the ERG blocker WAY-123,398 was applied, followed by linopirdine. ERG block has a minor effect on spiking rate, but not duration.
Figure 4-12
4.3: Discussion

The foregoing data make it clear that the NG108-15 M-like current can be separated kinetically and pharmacologically into two parts, of which one corresponds closely to the current generated by mErg1a channels expressed heterologously in CHO cells.

Four sets of criteria for identifying molecular counterparts of native currents are listed in Selyanko et al. (1999). These characteristics are: (1) the presence of mRNA transcripts and protein; (2) biophysical properties, including various kinetic measurements; (3) sensitivity to blockers; and (4) modulation by agonist action. For mErg1a as a basis for the NG108-15 slow current, the expression of the channels (point (1)) is covered in Selyanko et al. (1999). Collaborators in the Wellcome Laboratory for Molecular Pharmacology confirmed by RT-PCR the presence of mErg1 mRNA in both NG108-15 cells and mouse SCG neurones. Immunostaining for the mErg1 protein was strong in morphologically differentiated NG108-15 cells (which have large M-like currents), weak in undifferentiated cells (which have only very small M-like currents), and absent in mouse SCG neurones (which have only a standard fast M-current). Likewise, modulation by an agonist of M1 receptors (point (4)) is shown both for the NG108-15 slow current and for mErg1a in CHO cells (Selyanko et al. 1999). My own data, discussed here, address points (2) and (3): the kinetics and blocker sensitivities of mErg1a and the NG108-15 slow current.

Meves et al. (1999) have also separated the NG108-15 M-like current into a conventional M-current and an ERG current. Their work duplicates the pharmacological dissection approach described here and in Selyanko et al. (1999), but at physiological temperature. Meves et al. (1999) show that the ERG blocker E-4031 also partially blocks the fast portion of the current, demonstrating that WAY-123,398 is so far the only truly selective blocker for use on mixed M-like currents.

A novel aspect of this work (Meves et al. 1999; Selyanko et al. 1999) is that a HERG-related current, mErg1a, appears to provide a kinetic and pharmacological counterpart to a standing current seen at somewhat depolarised potentials in NG108-15 cells. It is also evident as a slow component of current relaxations. Channels in the ERG family have commonly been studied under conditions eliciting a transient current, and it is only recently that the effects of channel-blocking drugs have led researchers to look for other manifestations of ERG currents. The NG108-15 mErg1a-like current shows the steady-state inactivation typical of the ERG family, but still has a substantial number of channels open at voltages negative to the action potential threshold.
Depending on how the measurement was made, some 70% of the current in NG108-15 cells over the voltage range -20 to -50 mV was produced by channels whose macroscopic properties closely resembled those of mErg1a expressed in CHO cells. I found a 72% contribution of the slow current in a sample of 16 cells which had visibly slow relaxations of the whole current at -50 mV. In a total of 65 cells, I established an average slow current contribution of 70%. Pooled results for 86 cells, in Selyanko et al. (1999), yielded a 67% contribution of the slow current. A. A. Selyanko also found 15 differentiated NG108-15 cells, normal in other respects, in which only a slow current was present. For the standing current, a sample of 19 cells (including the 16 referred to above) showed a 66% contribution of the slow component, as assessed by the decrease in standing current at -20 mV in the presence of 10 µM WAY-123,398.

Because of gaps in the literature, much of the comparison data for mErg1 relates to the better-studied HERG channel. mErg1a is 96% identical to HERG in protein sequence, and >99% identical over the region including the transmembrane segments (London et al. 1997). Similarly, I have supplemented published data on NG108-15 cells by information on neuroblastoma lines analogous to the neuronal parent of NG108-15, and other neuroblastoma hybrids.

**Kinetics**

I studied deactivation of mErg1a in CHO cells at -110 to -10 mV, from a holding potential of 0 mV. I found two time constants of 50-460 and 450-4000 ms (plotted in Figure 4.7). London et al. (1997) obtained closely similar results in *Xenopus* oocytes, for mErg1a relaxations at -100 to -30 mV from a holding potential of +20 mV. Their graph shows deactivation ts of about 100-600 and 300-4000 ms. London et al.'s (1997) deactivation protocol evokes tail currents with peak amplitudes several times larger than the current at +20 mV, just as my tail currents peak considerably higher than the holding current at 0 mV. It looks as if oocytes and CHO cells present a very similar mErg1a channel complex in their membranes. Deactivation properties of mErg1a in oocytes also closely resemble those of HERG (London et al. 1997).

Kinetically, a striking resemblance emerges between mErg1a, expressed in CHO cells, and the slow fraction of the NG108-15 M-like current. The match holds good for the pattern of inactivation shown by the current/voltage curves, the magnitudes of the two ts fitted to the deactivations, and the relative contributions of the deactivation processes corresponding to
these at different voltages (Figures 4.6 and 4.7). No channel outside the ERG family has displayed such pronounced inactivation/de-inactivation behaviour.

My recordings of mErg1a were made using internal and external solutions with roughly physiological potassium concentrations. Numerous kinetic data have been reported for the human homologue HERG, but I shall not make comparisons with these because they were obtained at high external potassium concentrations (e.g. Trudeau et al. 1995). High external potassium levels shift the potassium reversal potential in a positive direction, enlarging and separating tail currents at strongly negative voltages, where properties can be studied with less hindrance from inactivation. High external potassium has been noted to affect inactivation (Wang et al. 1996b) and perhaps activation of HERG, leading to increased overall current (Wang et al. 1997a). Numaguchi et al. (2000) suggest that raised external potassium opposes a tonic channel block by external sodium, leading to increased HERG current amplitudes in high potassium. Physiological potassium concentrations highlight differences in current in the more positive voltage range relevant to action potential generation, and are found in papers discussing clinical (Sanguinetti et al. 1995) and pharmacological aspects of HERG (e.g. Busch et al. (1998) on azimilide).

**Blockers**

WAY-123,398 blocked the mErg1a and the NG108-15 slow M-like current with an IC$_{50}$ of 0.3 µM. There is no reported IC$_{50}$ for cloned ERG channels from any species to compare with this. On native currents, researchers often cite effects of single doses. The only published concentration-response data relate to inhibition of the "delayed rectifier" in cat ventricular myocytes (presumably feline ERG) by WAY-123,398 with an IC$_{50}$ around 0.1 µM (Spinelli et al. 1993).

With azimilide, I obtained low-micromolar IC$_{50}$ values, 6 µM on mErg1a and 8 µM on the slow portion of the M-like current. For comparison, Busch et al. (1998) found that azimilide (NE-10064) inhibited cloned HERG channels with an IC$_{50}$ of 5.2 µM at a stimulation frequency of 1 Hz, and 1.4 µM at 0.1 Hz (Busch et al. 1998). This decrease of block with increasing channel activation amounts to reverse use-dependence; I did not test for such phenomena on mErg1a or the NG108-15 current.

Overall, the similarity between blocker sensitivities of mErg1a and the NG108-15 slow current strongly supports the identity of the two currents. Four drugs, WAY-123,398, azimilide, THA and TEA, block the two currents at similar concentrations. Linopirdine at 30 µM produces a
small inhibition in both cases, but not enough to justify estimating an IC₅₀. Additionally, I
tested 10 μM WAY-123,398 on two types of cloned channels in CHO cells: mKv1.2 and
KCNQ2+KCNQ3 currents (n=3 for both) showed no inhibition, supporting the selectivity of
this compound.

Firing
My results on NG108-15 cells suggest that only a small increase in spiking rate, with no
increase in the duration of spiking activity, results from selective loss of the mErg1a-like
current. In these cells, a current apparently identical to the classic sympathetic M-current
acts within a short time as the major influence on accommodation.

A potassium current comparable to the slow NG108-15 current was also reported from
human and mouse neuroblastoma cells (Arcangeli et al. 1995). In a hybrid cell line derived
from mouse neuroblastoma and rat dorsal root ganglion cells, this "inward rectifier" current
shared the pharmacology of HERG, but had an even smaller outward current component
(Faravelli et al. 1996). Chiesa et al. (1997) proposed that ERG channels play a role in spike
frequency accommodation, since in this line, at room temperature, suppressing the ERG
current with WAY-123,398 led to increased firing frequencies during a sustained
depolarisation. Schönherr et al. (1999) extended this scenario with a human leukaemic cell
line and HERG mutants expressed in oocytes. The time taken to achieve a steady-state
ERG current during repetitive depolarisations depends on the voltage, and can be quite long.
Conceivably, tens of spikes might occur before the sustained ERG current becomes large
enough to inhibit further spiking.
Chapter 5: KCNQ2, KCNQ3 and the fast NG108-15 M-like current

5.1: Introduction – KCNQ channels

The KCNQ family of channels is closely related to the Shaker-type Kv channels, with other potassium channels such as the eag group being more distant on the phylogenetic tree (Derst & Karschin 1998). I shall give a brief history of the discovery of this group of channels.

An untypical potassium channel subunit with one transmembrane region known as I\textsubscript{K\theta} or minK (minimal K channel protein), and later as KCNE1, was found to mediate the cardiac slow delayed rectifier (Folander et al. 1990). It emerged subsequently that minK does not form channels on its own. Genetic research determined that the commonest cause of long QT syndrome (see Chapter 4) is mutation of a gene initially classified as a “Kv” channel because of a substantial amino acid similarity to Shaker (Wang et al. 1996a). This channel, KvLQT\textsubscript{1}, interacts with minK in Xenopus and mammalian expression systems (Sanguinetti et al. 1996; Barhanin et al. 1996). The ability of Xenopus oocytes to give currents when the oocytes are injected with minK message is caused by the presence of a Xenopus homologue of KvLQT\textsubscript{1} (Sanguinetti et al. 1996).

KvLQT\textsubscript{1}, despite its typical 6-transmembrane-segment structure and close relationship to the Kv channels, shares little pharmacology with other channel types. The chromanol compound 293B blocks the native I\textsubscript{K\theta} and cloned KvLQT\textsubscript{1} channels (Busch et al. 1996; Bleich et al. 1997), acting on the KvLQT\textsubscript{1} subunit (Loussouarn et al. 1997). 293B is inactive against HERG (Busch et al. 1997b). The HERG-blocking antiarrhythmics E-4031 and dofetilide are without effect on KvLQT\textsubscript{1} (Yang et al. 1997). The benzodiazepine compound L-364,373 activates the cardiac I\textsubscript{K\theta}, and KvLQT\textsubscript{1} (in the absence of minK) in oocytes (Salata et al. 1998).

The next family member was first identified in the nematode C. elegans and named nKQT\textsubscript{2} (Wei et al. 1996). Geneticists studying epilepsy discovered a mammalian homologue, KCNQ2, and a further family member, KCNQ3 (Biervert et al. 1998; Charlier et al. 1998; Singh et al. 1998; Stoffel & Jan 1998). The effects of damage to the KCNQ2 or KCNQ3 genes are comparatively mild: a rare condition called benign familial neonatal convulsions (BFNC), with seizures during the first weeks of life and usually no continuing epileptic syndrome. KCNQ2 mutations do not appear to contribute to any other epileptic syndromes besides BFNC (Steinlein et al. 1999).
When expressed individually in *Xenopus* oocytes, KCNQ2 and KCNQ3 produce small potassium currents, but coexpressing KCNQ2 and KCNQ3 gives currents 15-fold larger than those from the individual subunits. Both genes are expressed principally in the brain. In mouse brain, KCNQ3 expression lags behind KCNQ2 until postnatal day 30 (Tinel et al. 1998).

Pharmacologically, KCNQ2 is not affected by the KCNQ1 (KvLQT1) blocker 293B or the KCNQ1 activator L-364,373. However, the benzodiazepine L-735,821 blocks both KCNQ1 and KCNQ2 (Tinel et al. 1998). The anticonvulsant retigabine may owe its activity to opening of KCNQ2+KCNQ3 channels (Rundfeldt & Netzer 2000). Other agents inhibiting KCNQ2 are linopirdine and tetraethylammonium (see next section).

A further member of the family, KCNQ4, is genetically linked to deafness. KCNQ4, like KCNQ2, produces larger currents when coexpressed with KCNQ3 in *Xenopus* oocytes (Kubisch et al. 1999). A KCNQ5 has now been reported by two groups (Lerche et al. 2000; Schroeder et al. 2000). No disease has yet been firmly linked to KCNQ5 mutations, but a contribution to epileptic disorders is feasible.

**KCNQ channels in SCG and NG108-15 cells**

The fast portion of NG108-15 M-like current closely resembles the mouse sympathetic M-current (Selyanko et al. 1999). Wang et al.’s (1998a) identification of a KCNQ2+KCNQ3 heteromultimer as the molecular basis of the sympathetic M-current therefore also gave us a likely candidate for the NG108-15 fast current.

While McKinnon’s group (Dixon & McKinnon 1996) found no potassium channel mRNA expression levels that paralleled the pattern of M-current expression in rat sympathetic ganglia, they recently reported that the ganglionic expression of KCNQ2 did match the pattern of M-current expression (Wang et al. 1998a). KCNQ3 mRNA was present in all the ganglia tested, but higher levels of KCNQ2 mRNA distinguished the M-current-containing superior cervical ganglion from the coeliac and superior mesenteric ganglia, which do not have M-currents (Dixon & McKinnon 1996). Further, the small currents produced by KCNQ3 expressed on its own in heterologous systems suggest that the presence of both channel proteins is necessary for the emergence of an M-current. Kinetic data supported Wang et al.’s (1998a) identification, since activation and deactivation kinetics of oocyte-expressed KCNQ2+KCNQ3 currents resembled those of the M-current. Pharmacological findings added persuasive evidence, since the KCNQ channels were sensitive to low concentrations of the M-current blocker linopirdine and its analogue XE991. Eag-family currents, and Kv1.2 and
Kv4.3 (exemplifying delayed rectifiers and A-currents, respectively) were much less sensitive to these drugs (Wang et al. 1998a). An initial proposal of KCNQ2 as the M-channel (Wang et al. 1998b) was revised in Wang et al. (1998a) to acknowledge that the TEA sensitivity of the native M-current could only be accounted for by invoking a KCNQ2+KCNQ3 heteromultimer. KCNQ2 is readily inhibited by 1 mM TEA; KCNQ3 is much less sensitive, and the heteromultimer current is intermediate in sensitivity (Yang et al. 1998), like the native M-current.

Following Wang et al.'s (1998a) report, I examined the properties of the KCNQ2+KCNQ3 heteromultimer expressed in CHO cells, for comparison with the M-current (Selyanko et al. 1999) and the NG108-15 fast current (present work). For this, I expressed the KCNQ channels used by Wang et al. (1998a), human KCNQ2 and rat KCNQ3, separately and together. The human KCNQ2 sequence is 841 amino acids in length, with molecular weight 92.5 kilodaltons. The rat KCNQ3 sequence is 873 amino acids long, with molecular weight 97 kilodaltons (data from the Swiss Institute of Bioinformatics TrEMBL database). Wang et al. (1998a) found 95% amino acid sequence identity between rat KCNQ3 and a partial human KCNQ3 clone. NG108-15 cells probably express the mouse homologues, derived from the mouse neuroblastoma parent of this line. I do not have sequence information on these, but rat/mouse protein homology can be expected to be close.
5.2: Results

Heterologously-expressed KCNQ channels and the NG108-15 fast current

As described in Chapter 4, NG108-15 currents recorded 7-14 days after plating had a standing M-like current of about 0.5-1.5 nA when holding at -20 mV. About 30% of this amplitude was composed of a fast-deactivating current, resistant to WAY-123,398, that resembled the classic sympathetic ganglion M-current. After WAY-123,398 block of the slow NG108-15 current, the fast current yields kinetic and pharmacological data, which I compare here with a cloned counterpart. I expressed Dr. D. McKinnon’s clones of human KCNQ2 and rat KCNQ3 in CHO hml1 cells (see Methods) by transfection with LipofectAmine Plus. I expressed these subunits either separately or together in equal proportions by weight.

There was a striking difference in the sizes of currents produced by the KCNQ subunits. KCNQ2+KCNQ3 currents expressed readily: of the cells marked by CD8 expression (see Methods), an estimated ≥90% gave currents of 0.5-1 nA at -20 mV, one day after transfection. Two and three days after transfection, currents were even bigger, impairing recording stability. KCNQ2 expressed alone gave currents in about 80% of marked cells, with amplitudes of 0.2-1 nA at -20 mV, when recorded two days after transfection. Useful KCNQ3 current amplitudes were only achieved three days after transfection; under 50% of marked cells had even 50 pA of current, and currents much above 200 pA at -20 mV were extremely rare.

Kinetics

KCNQ2, KCNQ3 and KCNQ2+KCNQ3 currents show delayed rectifier behaviour. I have characterised this in several ways. Figure 5.1 shows examples of the three protocols used, from three different cells. The uppermost record in Figure 5.1 was obtained with the classic M-current protocol deactivating from -20 mV. Current-voltage and conductance-voltage curves derived from such records enable the KCNQ currents to be compared with native M-type currents (see later). The second record in Figure 5.1 illustrates a protocol activating the channels from -80 mV, giving a more complete current/voltage relationship. Most useful of all is a compound protocol following an initial activation to +50 mV with sequential deactivations to a range of more negative potentials, and finally to -70 mV. This forms the bottom record in Figure 5.1. The inset of Figure 5.1 shows a magnified view of the final relaxations in this protocol. The amplitudes of these relaxations give a valuable “current/voltage”, or rather conductance/voltage, curve: all measurements are made at the same potential, so the driving force on the potassium charge-carrier is the same.
Figure 5.1: Examples of KCNQ2+KCNQ3 currents

(Top) M-current voltage protocol and KCNQ2+KCNQ3 current family recorded in a CHO cell.

(Centre) Activation protocol and KCNQ2+KCNQ3 currents.

(Bottom) Double-deactivation protocol and KCNQ2+KCNQ3 currents.

(Inset) Magnified tail currents from the double-deactivation protocol, seen on stepping back to −70 mV.
-20 mV  
-110 mV  

M-current protocol

-80 mV  

Activation protocol

+50 mV  

Double deactivation protocol

25 pA
100 ms

0.5 nA
1 nA
0.5 nA

Figure 5-1
**Reversal potential**

The solutions used for KCNQ channel recordings (110 mM potassium internally and 2.5 mM potassium externally) give a theoretical reversal potential of $-95.54 \text{ mV}$, as described in the Results section of Chapter 4. The NG108-15 fast current reversed at an average of $-74.5 \text{ mV}$ ($n=19$). The reversal potentials I have used for conductance calculation came from the largest available data-set in each case. For KCNQ2+KCNQ3, $V_{\text{rev}}$ was $-78.2 \text{ mV}$ for eight deactivations from $-20 \text{ mV}$ (six deactivations from $+50 \text{ mV}$ gave a similar value of $-76.8 \text{ mV}$).

For KCNQ2 and KCNQ3 expressed separately, $V_{\text{rev}}$ values came from deactivations after a step to $+50 \text{ mV}$. $V_{\text{rev}}$ was $-82.2 \text{ mV}$ ($n=6$) for KCNQ2, and $-73.8 \text{ mV}$ ($n=7$) for KCNQ3. In the cases of KCNQ2 and KCNQ2+KCNQ3, the overall deviation from the calculated value is similar to that for mErg1a, and presumably reflects the same sources of error, including some 7.3 mV of liquid junction potential. KCNQ3, recorded under the same conditions as the other channels, showed a more positive reversal potential, commonly evident during recording as a change in the direction of tail currents at a nominal step potential of $-70 \text{ mV}$. While the influence of potassium build-up around the cell would be less with the small currents produced by this channel type, contamination by leak currents is proportionally greater, and may explain a reversal potential error. Tiny outward current relaxations close to the true reversal potential of KCNQ3 could be concealed by even a small transient inward leak, such as might occur through non-potassium-specific channels that reverse more positively.

**Conductance**

I have plotted two conductance/voltage curves for each type of KCNQ channel tested. One curve is derived from the back-fitted amplitude of relaxations at $-70 \text{ mV}$. The other is calculated from mean end-of-step amplitudes of currents activated from $-80 \text{ mV}$, corrected for driving force by the formula given in Chapter 3.2. All data-sets are normalised to the value at $+20 \text{ mV}$, for convenience with some incomplete data sets and to standardise properties with respect to KCNQ3, which shows inactivation at more positive potentials.

Figure 5.2 shows the data for KCNQ2+KCNQ3 coexpressed currents. Maximum conductance in each case reaches 1.1, where the value at $+20 \text{ mV}$ is set to 1. The slope of the curve (mV/e-fold change in conductance) is 12.5 for the activation steps, and 12.7 for the deactivation steps. The largest discrepancy is the less negative half-activation potential (-8 mV) measured for the activation steps, compared with that (-20 mV) for the deactivations. The problem is that in both sets of data, the lack of series resistance compensation on the large KCNQ2+KCNQ3 currents will have prevented the more positive command potentials.
being reached. Some later recordings with series resistance compensation (not shown) do
however provide evidence for slight inactivation of the current at very positive potentials,
which is however promptly removed on modest hyperpolarisations. The deactivation steps
are measured at −70 mV and inactivation is expected to have been removed from all but the
most positive. A $V_{1/2}$ of −20 mV, obtained from this data set, should be regarded as an interim
approximation.

Figures 5.3 and 5.4 show the equivalent data for KCNQ2 and KCNQ3, respectively. Here,
the two methods of calculating conductance give closely similar results. KCNQ2 shows half­
activation voltages −16 and −14 mV, and slope 13 and 12 mV, from deactivation and
activation protocols, respectively. In Figure 5.4, because of the inactivation of KCNQ3
currents, I fitted Boltzmann curves only up to the normalisation point at +20 mV, and obtained
a correspondingly negative half-activation voltage of −26 or −23 mV. The slopes were 11 and
8 mV for deactivation and activation data, respectively. Slope factors relate to the gating
behaviour of the channels, in particular to the movement of charges in the voltage sensor.
Given that both KCNQ2 and its heteromultimers with KCNQ3 show slopes around 12, this
might be the expected slope for KCNQ3 as well. However, KCNQ3 currents were very small,
and the relaxation amplitudes at −70 mV (source of the 11 mV slope factor) were often only a
few picoamps. The larger currents measured during activation steps (producing the 8 mV
slope) gave a much smoother, and probably more reliable, curve.
Figure 5.2: Two conductance/voltage curves for KCNQ2+KCNQ3

(Top) Averaged normalised amplitude, ± s.e.m., of KCNQ2+KCNQ3 deactivation tails at −70 mV (see Figure 5.1 inset), against potential during the preceding step.

Boltzmann fit parameters:

- Normalised $G_{\text{max}} = 1.07 \pm 0.01$
- $V_{50} = -19.79 \pm 0.52$ mV
- $k = 12.72 \pm 0.44$ mV

(Bottom) Averaged normalised conductance, ± s.e.m., calculated from KCNQ2+KCNQ3 activations (see Figure 5.1 centre), against step potential.

Boltzmann fit parameters:

- Normalised $G_{\text{max}} = 1.12 \pm 0.01$
- $V_{50} = -8.32 \pm 0.65$ mV
- $k = 12.48 \pm 0.53$ mV
Figure 5-2

KCNQ2+KCNQ3 (n=4),
deactivated to -70 mV

KCNQ2+KCNQ3 (n=8),
activated from -80 mV
Figure 5.3: Two conductance/voltage curves for KCNQ2

(Top) Averaged normalised amplitude, ± s.e.m., of KCNQ2 deactivation tails at −70 mV (as in Figure 5.1 inset), against potential during the preceding step.
Boltzmann fit parameters:
- normalised $G_{\text{max}} = 1.10 \pm 0.01$
- $V_{\text{h}} = -15.51 \pm 0.56$ mV
- $k = 13.07 \pm 0.47$ mV

(Bottom) Averaged normalised conductance, ± s.e.m., calculated from KCNQ2 activations (as in Figure 5.1 centre), against step potential.
Boltzmann fit parameters:
- normalised $G_{\text{max}} = 1.06 \pm 0.01$
- $V_{\text{h}} = -14.01 \pm 0.67$ mV
- $k = 11.74 \pm 0.56$ mV
KCNQ2 (n=5) deactivated to -70 mV

KCNQ2 (n=9) activated from -80 mV

Figure 5-3
Figure 5.4: Two conductance/voltage curves for KCNQ3

(Top) Averaged normalised amplitude, ± s.e.m., of KCNQ3 deactivation tails at −70 mV (as in Figure 5.1 inset), against potential during the preceding step, fitted up to the normalisation point at +20 mV.
Boltzmann fit parameters:
\[ V_\theta = -26.43 \pm 1.46 \text{ mV} \]
\[ k = 11.43 \pm 1.31 \text{ mV} \]

(Bottom) Averaged normalised conductance, ± s.e.m., calculated from KCNQ3 activations (as in Figure 5.1 centre), against step potential.
Boltzmann fit parameters:
\[ V_\theta = -22.65 \pm 1.03 \text{ mV} \]
\[ k = 7.59 \pm 0.91 \text{ mV} \]
KCNQ3 (n=5), deactivated to -70 mV

KCNQ3 (n=6), activated from -80 mV

Figure 5-4

membrane potential (mV)
NG108-15 current dissection

As indicated in Chapter 4, the two constituents of the whole NG108-15 M-like current can be isolated in two ways. The KCNQ-like portion appears as the fastest of three exponential relaxation components, or as the residual current in 10 µM WAY-123,398. Figure 5.5 combines a recording of KCNQ2+KCNQ3 (as in Figure 5.1) and of the NG108-15 current recorded in the presence of 10 µM WAY-123,398 (from Figure 4.2) for comparison. The use of a subtraction method for obtaining the slow current unfortunately results in all my NG108-15 fast currents being recorded on 6-second steps, so it should be borne in mind that the time-scale of this NG108-15 fast current record is only half that of the KCNQ2+KCNQ3 example. Nevertheless, the resemblance of the current profiles is evident both in the early completion of the relaxation and in the spacing of the currents at the end of the steps.

Figure 5.6 shows the similarity of the current/voltage relationships for KCNQ2+3 in CHO cells and for the fast current revealed by WAY-123,398 treatment of NG108-15 cells. These data were obtained using the M-like current protocol (deactivations from −20 mV for 1 second on the KCNQ2+KCNQ3 current and 6 seconds for the NG108-15 current). Instantaneous currents are from tail-currents back-fitted with an appropriate number of exponential decay components. The end-of-step values are mean amplitudes, as before.

A single exponential fit was visually satisfactory for NG108-15 deactivations in 10 µM WAY-123,398, but did not coincide with the initial part of KCNQ2+KCNQ3 current relaxations in CHO cells, as illustrated for a single trace from each current type in Figure 5.7a. The time constants from these data are plotted in Figure 5.7b, and show similar magnitudes for the two current types, with the single NG108-15 time constants being intermediate in size between the double KCNQ2+KCNQ3 ones. Figure 5.8 illustrates the averaged reciprocals of the time constants for deactivation from −20 mV. These show a decline towards more positive potentials, rather than the U-shaped curve seen in Robbins et al. (1992). The bottom plot in Figure 5.8 is derived from single-exponential fits to relaxations from +50 mV (using the protocol shown at the bottom of Figure 5.1) in five KCNQ2+KCNQ3 currents. These currents were large at +50 mV, ranging from 1.4 to 3.5 nA and averaging 2.4 nA. There is consequently some shortfall in the membrane potential achieved, since series resistance was not compensated. Notwithstanding, a U-shaped curve does appear here, with a minimum at about −30 mV, close to the $V_{th}$ of −20 mV derived from conductance measurements based on the maximum amplitudes of deactivating currents at −70 mV (Figure 5.4).
Figure 5.5: KCNQ2+KCNQ3 and the NG108-15 fast current compared

(Above) Voltage protocol and KCNQ2+KCNQ3 current deactivating from -20 mV.

(Below) Voltage protocol and WAY-123,398-resistant current in NG108-15, deactivating from -20 mV. Note that the scale of the time axis is half that of the upper record.
KCNQ2+KCNQ3 in CHO hm1

WAY-123,398-resistant current in NG108-15

Figure 5-5
Figure 5.6: Partial current/voltage relationships for KCNQ2+KCNQ3 and the NG108-15 fast current

(Top) Back-fitted instantaneous and end-of-step (at ~1 second) current amplitudes, ± s.e.m., for KCNQ2+KCNQ3 currents in eight CHO cells.

(Bottom) Back-fitted instantaneous and end-of-step (at ~6 seconds) current amplitudes, ± s.e.m., for the fast (WAY-123,398-insensitive) current in nineteen NG108-15 cells.
KCNQ2+KCNQ3, n=8
- end-of-step
- instantaneous

NG108-15 fast, n=19
- end-of-step
- instantaneous

Figure 5-6
Figure 5.7: Deactivation kinetics of KCNQ2+KCNQ3 and NG108-15 fast currents

Figure 5.7a: (Top) Initial region of a relaxation at −50 mV (from −20 mV) for a KCNQ2+KCNQ3 current in a CHO hml cell, with one- and two-exponential fits superimposed. A one-component fit is inadequate for this current, while a two-component fit matches it well.

(Bottom) Initial region of a relaxation at −50 mV (from −20 mV) for an NG108-15 fast current, in the presence of 10 µM WAY-123,398, with a one-exponential fit superimposed.

Figure 5.7b: (Top) $\tau \pm$ s.e.m. versus voltage from a two-exponential fit to KCNQ2+KCNQ3 current deactivations from −20 mV in eight CHO cells.

(Bottom) $\tau \pm$ s.e.m. versus voltage from a single-exponential fit to NG108-15 fast (WAY-123,398-resistant) current deactivations from −20 mV in nineteen cells.
2-exponential fit: 
τs about 30 and 100 ms

1-exponential fit: 
τ about 60 ms

NG108-15 fast current 
in WAY-123,398

Figure 5-7a
Figure 5-7b

**KCNQ2+KCNQ3, n=8**
- ▲ slow \( \tau \)
- ■ fast \( \tau \)

**NG108-15 fast, n=19**
- ■ mean \( \tau \)
Figure 5.8: Deactivation as $1/\tau$ for KCNQ2+KCNQ3 and NG108-15 fast currents

(Top) $1/\tau \pm \text{s.e.m.}$ versus voltage from a single-exponential fit to nineteen NG108-15 fast (WAY-123,398-resistant) current deactivations from $-20$ mV.

(Centre) $1/\tau \pm \text{s.e.m.}$ versus voltage from a two-exponential fit to KCNQ2+KCNQ3 current deactivations from $-20$ mV in eight CHO cells.

(Bottom) $1/\tau \pm \text{s.e.m.}$ versus voltage from a single-exponential fit to KCNQ2+KCNQ3 current deactivations from a nominal command voltage of $+50$ mV in five CHO cells (note that the more positive command voltages will not have been achieved: see Discussion).
NG108-15 fast current relaxations from -20 mV

KCNQ2+KCNQ3 relaxations from -20 mV

KCNQ2+KCNQ3 relaxations from +50 mV

Figure 5-8
Pharmacology

The slow portion of the NG108-15 M-like current was blocked with 10 μM WAY-123,398, and the drugs of interest were applied cumulatively in doses following a 1,3,10,30... pattern, giving intervals of about 0.5 log unit. The relaxation at -50 mV (during a 6-second step from a holding potential of -20 mV) was assessed for a single control trace, and for a trace showing a stable current amplitude at each concentration of the blocker. Using Clampfit, one exponential was back-fitted to NG108-15 fast currents, and two to KCNQ currents in CHO cells (see Methods). From the mathematically-corrected relaxation amplitudes, current inhibition was calculated, plotted, and fitted as described for mErg1a.

Figure 5.9 demonstrates the selectivity of linopirdine, at low concentrations, for KCNQ2+KCNQ3 currents and the NG108-15 fast current. Doses from 3 to 30 μM linopirdine cause a substantial, concentration-dependent block of these currents. For comparison, linopirdine up to 30 μM had only a modest effect on relaxations of mErg1a. The record shown for mKv1.2 is a ramp current, which is only about half-inhibited by 100 μM linopirdine dissolved in dimethylsulphoxide. Similar results were obtained in a further 3 cells with this protocol. [Brief tests suggested that linopirdine was slightly more potent against the NG108-15 fast current (n=3, not shown) when dissolved in DMSO rather than in 0.1N HCl. DMSO alone at equivalent concentrations caused no inhibition. I did not test aqueous acidic linopirdine stocks against mKv1.2, but an equal or lesser effect seems likely on this basis].

Figure 5.10 shows concentration/response data for linopirdine, azimilide and tetraethylammonium on the KCNQ2+KCNQ3 current expressed in CHO hml1 cells. I have added the data for TEA on KCNQ2 and KCNQ3 separately, for comparison. Figure 5.11 gives the equivalent data for the NG108-15 fast current. Table 3 summarises the block of the NG108-15 fast current and of KCNQ currents by linopirdine, azimilide and TEA. Linopirdine and azimilide are similar in their effects on KCNQ2, KCNQ3 and KCNQ2+KCNQ3 currents; only TEA distinguishes between the different KCNQ channels. The TEA sensitivity of the NG108-15 fast current is compatible with a composition of KCNQ2+KCNQ3 heteromultimers, perhaps with a relatively high contribution of KCNQ2; the shallow slope for the NG108-15 fast current may suggest a variable subunit stoichiometry.

I also fitted curves to the drug inhibition data for individual cells, and used a two-tailed Student’s t-test (in Excel) for comparison between the KCNQ channel currents and the NG108-15 fast current, obtaining the P values shown in the lower part of Table 3. No P values of less than 0.05 (which would suggest a significant difference between data for
KCNQ currents and the NG108-15 fast current) were obtained. Cross-comparison between KCNQ channel current and NG108-15 slow current inhibition by azimilide and TEA also gave no significant differences. However, see also comments (p.122) about Table 2, and (p.177) in Chapter 6.

As shown in Figure 4.12 and discussed in Chapter 4, firing of NG108-15 cells in response to an applied depolarisation at room temperature was prolonged by linopirdine at a KCNQ channel-blocking dose of 30 μM. By contrast, the ERG channel blocker WAY-123,398 had only a marginal effect on firing rate under these conditions.
**Figure 5.9: Selectivity of linopirdine**

The effect of linopirdine on examples of four currents, with the relevant voltage protocol shown above each one.

(Above left) KCNQ2+KCNQ3. Decrease in the holding current at −20 mV and the relaxation at −50 mV, by 3 μM linopirdine. Most of the standing current and relaxation removed by 30 μM linopirdine.

(Above right) NG108-15 fast current, in the presence of 10 μM WAY-123,398. Big decrease in the holding current at −20 mV and the relaxation at −50 mV, by 3 μM linopirdine. Most of the standing current and relaxation removed by 30 μM linopirdine.

(Below left) mErg1a expressed in a CHO hm1 cell. Small effect of doses from 3 to 30 μM linopirdine on the relaxation at −50 mV; negligible effect on the standing current at 0 mV. Similar effect on relaxations in a further 2 cells.

(Below right) mKv1.2 in an MK2 cell. ~50% inhibition of mKv1.2 current (evoked by a ramp from −78 to +52 mV) by a large dose of 100 μM linopirdine dissolved in dimethylsulphoxide. Similar result in a further 3 cells.
Figure 5-9

KCNQ2+KCNQ3

NG108-15 fast current

mErg1a

mKv1.2

-20 mV

-50 mV

100 pA

0.25 s

100 pA

1 s

0 mV

-50 mV

25 pA

2 s

2 nA

0.5 s

3 nM linopirdine

100 nM linopirdine

10 nM linopirdine dissolved in DMSO

30 nM linopirdine
Figure 5.10: Concentration/response curves on KCNQ channels

All data are plotted as mean inhibition ± s.e.m.

(Top) Linopirdine (n=4-5) on KCNQ2+KCNQ3
Hill equation fit parameters:
\( Y_{\text{max}} = 100\% \)
\( IC_{50} = 5.10 \pm 0.61 \mu M \)
\( n_H = 0.95 \pm 0.10 \)

(Centre) Azimilide (n=4) on KCNQ2+KCNQ3
Hill equation fit parameters:
\( Y_{\text{max}} = 100\% \)
\( IC_{50} = 73.55 \pm 8.94 \mu M \)
\( n_H = 0.68 \pm 0.06 \)

(Bottom) TEA (n=4) on KCNQ2 alone
Hill equation fit parameters:
\( Y_{\text{max}} = 100\% \)
\( IC_{50} = 0.29 \pm 0.02 \text{ mM} \)
\( n_H = 0.93 \pm 0.06 \)

TEA (n=4) on KCNQ2+KCNQ3
Hill equation fit parameters:
\( Y_{\text{max}} = 100\% \)
\( IC_{50} = 3.82 \pm 0.20 \text{ mM} \)
\( n_H = 0.76 \pm 0.03 \)
Figure 5.11: Concentration/response curves on the NG108-15 fast current

All data are plotted as mean inhibition ± s.e.m.

(Top) Linopirdine (n=4)
Hill equation fit parameters:
\[ Y_{\text{max}} = 100\% \]
\[ IC_{50} = 1.07 \pm 0.23 \mu M \]
\[ n_H = 1.21 \pm 0.28 \]

(Centre) Azimilide (n=3)
Hill equation fit parameters:
\[ Y_{\text{max}} = 100\% \]
\[ IC_{50} = 35.53 \pm 3.81 \mu M \]
\[ n_H = 1.04 \pm 0.12 \]

(Bottom) TEA (n=3)
Hill equation fit parameters:
\[ Y_{\text{max}} = 100\% \]
\[ IC_{50} = 1.16 \pm 0.30 \text{ mM} \]
\[ n_H = 0.47 \pm 0.07 \]
Table 3: Block of the NG108-15 fast current and of KCNQ currents by linopirdine, azimilide and TEA

All IC\textsubscript{50} values are quoted ± s.e.m., firstly, as calculated on averaged inhibition data:

<table>
<thead>
<tr>
<th>Drug</th>
<th>NG108-15 fast</th>
<th>KCNQ2 alone</th>
<th>KCNQ2+KCNQ3</th>
<th>KCNQ3 alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linopirdine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC\textsubscript{50} ± s.e.m.</td>
<td>1.1 ± 0.2 µM</td>
<td>3.7 ± 0.1 µM</td>
<td>5.1 ± 0.6 µM</td>
<td>4.8 ± 0.6 µM</td>
</tr>
<tr>
<td>n\textsubscript{H}</td>
<td>1.2</td>
<td>1.2</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>n (cells)</td>
<td>4</td>
<td>4</td>
<td>4-5</td>
<td>4</td>
</tr>
<tr>
<td>Azimilide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC\textsubscript{50}</td>
<td>35.5 ± 3.8 µM</td>
<td>70.6 ± 4.8 µM</td>
<td>73.6 ± 8.9 µM</td>
<td>12.7 ± 1.8 µM</td>
</tr>
<tr>
<td>n\textsubscript{H}</td>
<td>1.0</td>
<td>1.2</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>n (cells)</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>TEA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC\textsubscript{50}</td>
<td>1.2 ± 0.3 mM</td>
<td>0.3 ± 0.02 mM</td>
<td>3.8 ± 0.2 mM</td>
<td>~142 mM</td>
</tr>
<tr>
<td>n\textsubscript{H}</td>
<td>0.5</td>
<td>0.9</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>n (cells)</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

and secondly from fitting data individually for each cell, averaging and applying a t-test:

<table>
<thead>
<tr>
<th>Drug</th>
<th>NG108-15 fast</th>
<th>KCNQ2 alone</th>
<th>KCNQ2+KCNQ3</th>
<th>P value, IC\textsubscript{50} KCNQ vs. NG fast</th>
<th>P value, IC\textsubscript{50} KCNQ2+3 vs. NG slow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linopirdine</td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>IC\textsubscript{50} ± s.e.m.</td>
<td>1.1 ± 0.38</td>
<td>5.88 ± 1.56</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n\textsubscript{H}</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>n (cells)</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Azimilide</td>
<td></td>
<td></td>
<td></td>
<td>0.40</td>
<td>0.06</td>
</tr>
<tr>
<td>IC\textsubscript{50}</td>
<td>42.67 ± 23.67</td>
<td>82.5 ± 32.69</td>
<td>0.40</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>n\textsubscript{H}</td>
<td>0.9</td>
<td>0.95</td>
<td>0.95</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>n (cells)</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>TEA</td>
<td></td>
<td></td>
<td></td>
<td>0.35 (KCNQ 2+3); 0.07 (KCNQ2)</td>
<td>0.30</td>
</tr>
<tr>
<td>IC\textsubscript{50}</td>
<td>1.62 ± 0.65</td>
<td>0.32 ± 0.09</td>
<td>6.98 ± 4.39</td>
<td>0.35 (KCNQ 2+3); 0.07 (KCNQ2)</td>
<td>0.30</td>
</tr>
<tr>
<td>n\textsubscript{H}</td>
<td>0.7</td>
<td>0.95</td>
<td>0.85</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>n (cells)</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
5.3: Discussion

The NG108-15 M-like current is now seen to comprise, in addition to a mErg1a-like current, another current which resembles that generated by KCNQ2+KCNQ3 heteromultimeric channels expressed in CHO cells.

As previously mentioned, diagnostic criteria for the identities of native currents are: (1) the presence of mRNA transcripts and protein; (2) biophysical properties, including various kinetic measurements; (3) sensitivity to blockers; and (4) modulation by agonist action (Selyanko et al. 1999).

Regarding KCNQ2+KCNQ3 as a basis for the NG108-15 fast current, the expression of the channels (point (1)) is partially covered in Selyanko et al. (1999). Collaborators in the Wellcome Laboratory for Molecular Pharmacology confirmed by RT-PCR the presence of KCNQ2 and KCNQ3 mRNAs in both NG108-15 cells and mouse SCG neurones. Immunostaining for the KCNQ channel proteins is currently under study. Modulation by an agonist of M₃ receptors (point (4)) has been shown for the NG108-15 fast current and the mouse SCG M-current (Selyanko et al. 1999), and for KCNQ channels in CHO cells (Selyanko et al. 2000). In a CHO cell environment, all the KCNQ channel types from KCNQ1 to KCNQ4 were readily inhibited by the M₃ receptor agonist oxotremorine methiodide.

My own data, discussed here, address points (2) and (3): the kinetics and blocker sensitivities of KCNQ2, KCNQ3, KCNQ2+KCNQ3 coexpressed currents and the NG108-15 fast current. As collaborative data show in Selyanko et al. (1999), the fast current in NG108-15 cells corresponds, in kinetics, pharmacology and capacity for muscarinic modulation, to the mouse sympathetic ganglion M-current. I show here that the behaviour of the NG108-15 fast current is also very similar to that of a heteromultimer of KCNQ2+KCNQ3 subunits.

I found that about 30% of the NG108-15 M-like current was produced by channels with macroscopic properties resembling those of KCNQ2+KCNQ3 expressed in CHO cells. The fast current contribution to relaxations at −50 mV was 33% in collaborative results for 86 cells (Selyanko et al. 1999). Other figures can be calculated from the Discussion section of Chapter 4, where the mErg1a-like current contribution is quantified. The consistent kinetic properties of the WAY-123,398-resistant current, and the possibility of blocking it fully with 30 μM linopirdine, confirm that only two current types are present, so the fast current contribution is 100% minus the slow current contribution.
Kinetics

The properties of deactivation tail currents compare well for KCNQ2+KCNQ3 and the WAY-123,398-resistant current in NG108-15 cells (examples in Figure 5.5 and averaged current/voltage data in Figure 5.6). The deactivation time constants plotted in Figure 5.7 are similar in magnitude for the cloned and the native current. The need for a two-exponential fit for KCNQ2+KCNQ3 currents in CHO cells, while a single exponential sufficed for the NG108-15 fast current, may relate in part to the different length of the deactivation steps. I commonly fitted only the first part of the 6-second step used on the NG108-15 M-like current. Still, the longer sampling interval, and the need to avoid NG108-15 transients while placing fitting cursors, may have concealed a fast component of the relaxation such that a two-exponential deactivation was approximated by a one-exponential fit.

I found the activation curve of the KCNQ2+KCNQ3 channel to be positively shifted relative to literature values for the sympathetic M-current and the NG108-15 M-like current. However, the results of Constanti & Brown (1981) and Robbins et al. (1992), with $V_m$ at $-44$ or $-45$ mV, were obtained at raised temperatures. It appears that the $V_m$ for the fast M-like current may occur at more positive potentials at room temperature.

Even though I have not been able to study the fast M-like current at strongly positive potentials, there is provisional evidence from deactivation time constants that its activation midpoint $V_m$ is positive to $-30$ mV at room temperature. This is because the reciprocal (mono-exponential) time-constants of Figure 5.8, plotted against step potential, do not show an upturn at the positive end of the voltage range used.

A U-shaped relationship of $1/\tau$ to step potential, as found for the whole NG108-15 M-like current in Robbins et al. (1992), and the sympathetic M-current in Constanti & Brown (1981), is an expected property of a channel whose opening and closing is determined by a single conformational transition. The closing rate of the channels falls off as voltage becomes more positive, and the opening rate increases rapidly, according to the exponential relationships: $\alpha = \alpha_0 \exp\left(\frac{ze}{2kT}(V-V_m)\right)$ and $\beta = \beta_0 \exp\left(-\frac{ze}{2kT}(V-V_m)\right)$, where $\alpha$ and $\beta$ are the opening and closing rate constants, respectively, $e$ is the elementary charge, and $z$ represents the charge transfer within the channel that leads to opening or closing (Adams et al. 1982). Further, the time constant $\tau$ for a current relaxation is the reciprocal of $(\alpha + \beta)$ (Hille 1984, p.47), so $1/\tau$ follows the U-shaped curve generated by averaging $\alpha$ and $\beta$. Since $\alpha$ and $\beta$ are functions of $V_m$ with opposite signs, the point where $\alpha = \beta$, and the sum of the two curves is minimal, falls at $V_m$. 
Figure 5.8 shows reciprocal plots of the time constants obtained for relaxations from $-20\, \text{mV}$ for both fast M-like and KCNQ2+KCNQ3 channels. Neither of these data-sets yields a U-shape over the range of potentials used. However, data from a single-exponential fit of KCNQ2+KCNQ3 current relaxations from $+50\, \text{mV}$ shows that $1/\tau$ starts to increase with voltage positive to about $-20\, \text{mV}$. This potential corresponds to the value of $V_{50}$ obtained from the deactivation-derived conductance/voltage curve in Figure 5.2. The U-shape is not well-maintained above $0\, \text{mV}$, probably because uncompensated series resistance has prevented the command voltages from being achieved. However, even if the step preceding deactivation reached only $+20\, \text{mV}$ rather than $+50\, \text{mV}$, it was consistent in each cell, and high enough to indicate the trend in the deactivation rate up to about $0\, \text{mV}$. The NG108-15 fast current has such a low $\tau$ at $-30\, \text{mV}$ that, on the basis of its general similarity to KCNQ2+KCNQ3 currents, this is likely to be close to the minimum. Thus, I have not been able to measure directly the $V_{50}$ for the NG108-15 fast current, but it is probably in the range of $-30$ to $-20\, \text{mV}$.

However, the assumption of a single opening/closing transition determining fast M-like current behaviour is probably an oversimplification. At best, it is an approximation to the behaviour of native currents which may not be reproduced in a non-neuronal cell type. Activation of the KCNQ currents in CHO cells (centre section of Figure 5.1) reproduces a common observation also visible in oocyte recordings (Wang et al. 1998a; Yang et al. 1998). KCNQ2, KCNQ3 and KCNQ2+KCNQ3 currents all show an initial sigmoidal phase of activation. Native M-currents reconstituted from channel recordings in sympathetic neurones by Selyanko et al. (1992) and in NG108-15 cells by Selyanko et al. (1995) show exponential activation. This suggests that some extra complexity may exist in the gating process of KCNQ channels when expressed heterologously. Only further experiment will determine whether it is connected with the make-up of heteromultimers, phosphorylation state of the channel, accessory subunits, or other post-translational modifications of the channels.

**Blockers**

Linopirdine block of the NG108-15 fast current (Table 3), with $IC_{50}$ 1.1 $\mu\text{M}$, was comparable with the 4-5 $\mu\text{M}$ obtained for KCNQ2, KCNQ3 and their mixtures. Linopirdine blocks KCNQ2+KCNQ3 currents in *Xenopus* oocytes with $IC_{50}$ 4.0 $\mu\text{M}$ (Wang et al. 1998), compared with 3.4 $\mu\text{M}$ (Lamas et al. 1997) or 7.0 $\mu\text{M}$ (Costa & Brown 1997) on the SCG M-current. My fitted Hill slopes for linopirdine block are close to unity, suggesting a simple channel/blocker interaction. Linopirdine is selective enough to discriminate KCNQ-like channels from other
clones: at 30 \( \mu M \) it had little effect on mErq1a, and even 100 \( \mu M \) linopirdine inhibited mKv1.2 by no more than half.

The IC\(_{50} \) of 35.5 \( \mu M \) for azimilide on the fast NG108-15 current falls within the 13-75 \( \mu M \) range found for KCNQ2, KCNQ3, and combinations in CHO cells (Table 3). The low Hill slope (0.7) on KCNQ2+3 might be interpreted as evidence for a mixed channel population, but the data-points do not look biphasic. In any case, this is not observed with the NG108-15 fast current, where the Hill slope is unity.

As stated in Chapter 4, the ERG blocking drug WAY-123,398 had no inhibitory effect on KCNQ2+KCNQ3 currents (n=3) at 10 \( \mu M \).

**TEA and stoichiometry**

Channel block by tetraethylammonium is interesting in relation to the precise molecular identity of M-type currents in various cells. KCNQ2 is particularly sensitive to TEA, KCNQ3 particularly insensitive, and the KCNQ2+KCNQ3 current intermediate in sensitivity. The high sensitivity of KCNQ2 is conferred by the presence of a tyrosine residue just downstream of the potassium-selective pore signature sequence GYG, as in TEA-sensitive channels in the Shaker group (Hadley et al. 2000). The TEA IC\(_{50} \) of 1.2 ± 0.3 mM on the fast NG108-15 current is slightly lower than the value of 3.8 ± 0.2 mM for KCNQ2+KCNQ3 channels (Table 3). The presence of KCNQ2 homomultimers in NG108-15 cells, or a higher proportion of KCNQ2 relative to KCNQ3 in the heteromultimer assemblies, is possible. The shallow Hill slope of 0.5 fitted to the NG108-15 fast current data is compatible with a mixed channel population, but I do not propose to draw conclusions from such a small sample.

Other investigators have expressed KCNQ2 and/or KCNQ3 subunits in another cell type, the tsA-201 strain of human embryonic kidney cells (Shapiro et al. 2000). They fitted Hill equations to KCNQ2 and (very incomplete) KCNQ3 data, obtaining Hill slopes close to 1. For KCNQ2+KCNQ3-cotransfected cells, Shapiro et al. (2000) obtained a Hill slope of 0.56 when they fitted a single Hill equation, suggesting non-homogeneous channel types. They preferred a fit of five Hill equations, to allow for all possible subunit combinations in the channel tetramer.

My own TEA data in CHO cells give Hill slopes 0.8 (KCNQ2+KCNQ3), and 0.9 (KCNQ2), a bit low to be taken as unity. My own fit to incomplete KCNQ3 data would suggest a Hill slope of 0.7. Data obtained by A.A. Selyanko and myself show Hill slopes markedly less than unity.
for TEA on both NG108-15 current constituents as well as on mErg1a and on the mouse ganglion M-current (Selyanko et al. 1999). The Hill slopes for TEA inhibition of KCNQ1 and KCNQ4 are close to 2, which is as yet unexplained in molecular terms (work by A.A. Selyanko in Hadley et al. 2000). Perhaps the block of KCNQ and ERG channels by TEA involves something other than a simple one-to-one binding reaction. No experimenters have used high enough TEA concentrations to show convincingly that KCNQ3 conforms to the one-subunit, unity-Hill-slope “rule”. I do not think that any of the data presently available for TEA block justify any conclusions about the composition of channels in a mixed transfection.

Overall, the sensitivity of KCNQ2+KCNQ3 to linopirdine, azimilide and TEA matches that of the NG108-15 fast current, strongly supporting the identity of the two currents.
Chapter 6: General discussion and conclusions

In summary, I have used the patch-clamp technique to dissect the M-like current in neuroblastoma-glioma hybrid cells, making particular use of kinetic measurements and channel-blocking agents. I conclude that channel-selective blockers, when backed up by kinetic and expression data, provided a particularly valuable tool for identifying M-like current constituents. My findings are that the M-like current consists partly of a current sensitive to WAY-123,398 and resembling that produced by mErg1a channels, and partly of a current sensitive to linopirdine and matching the properties of coexpressed KCNQ2+KCNQ3 subunits. These two blockers at low concentrations eliminate the whole M-like current, indicating that Kv1.2 and other channels are unlikely to contribute.

What has also emerged is that, while the sympathetic M-current has been commonly described as "non-inactivating", this term cannot be considered valid for the NG108-15 M-like current. Certainly, KCNQ2, KCNQ2+KCNQ3 and the fast component of the M-like current show no signs of inactivation at normal M-current recording potentials. However, the slow WAY-123,398-inhibited current, separated by data subtraction, shows unmistakable inactivation behaviour. It is nevertheless significant that mErg1a and the mErg1a-like NG108-15 current undergo rapid inactivation and de-inactivation, revealed only by paradoxical changes in current amplitude. This does not invalidate the slow inactivation seen in Kv1.2 as evidence against its participation in the M-like current.

To recapitulate, four classes of criteria have been specified as important in identifying the molecular counterparts of native currents (Seyanko et al. 1999): (1) the presence of mRNA transcripts and protein; (2) biophysical properties; (3) sensitivity to blockers; and (4) modulation by agonist action. The molecular determinants of these behaviours are localised to varying extents in the channel structure. The intracellular termini of channel subunits contain distinct sites which affect expression of the protein, some kinetic properties, and modulation, through membrane targeting, association with accessory subunits and the cytoskeleton, and phosphorylation. Most of these involve specific short peptide sequences. Channel gating depends on the S4 transmembrane voltage sensor and its interactions with the surrounding regions. Miller (1995) makes the point that drastically altered sensitivity to a particular blocker can be caused by variations in channel protein sequence having no effects on channel gating. Blocker sensitivity characteristically relates to amino acid residues in the extracellular mouth of the channel, which means that it can vary independently of kinetic properties. This is valuable for channel identification, because kinetic and pharmacological data provide two effectively separate lines of evidence.
I would also like to consider how closely particular types of property need to match between native currents and heterologously-expressed channels, for a firm identification to be made. I have certain reservations in respect of modulation. From a chemist's point of view, direct molecular interactions are more readily detected than indirect ones, so the most persuasive evidence of channel identity comes from properly-controlled experiments in which one substance binds with high affinity to another.

Molecular biology techniques can rigorously demonstrate the presence of a known channel. Messenger RNA can be visualised in a cell (by \textit{in-situ} hybridisation) or after extraction (as a Northern blot) by allowing it to bind to a radioactively-labelled nucleotide sequence. This approach can identify candidate channels. Immunocytochemistry techniques bind an antibody stably to the protein of interest. A second antibody is commonly used to improve visualisation. In this case, three molecules are stably bound to each other with high affinity. A further use of antibodies is for co-immunoprecipitation, as used to identify the complexes of alpha- and beta-subunits forming dendrotoxin-binding Kv1 channels in the brain (Rhodes et al. 1995). An occasional drawback is that these techniques require prior knowledge of the channel sought: at least a partial gene- or protein sequence is needed to create the oligonucleotide probe for the mRNA, or the antigen to raise the antibodies.

\textbf{Biophysical properties} such as kinetics and single-channel behaviour may conceivably be altered by intracellular interactions with cytoskeletal molecules and unidentified accessory subunits. This field is still in the empirical stage of development. However, kinetic and single-channel properties of cloned channels can provide a guide to the correct subunit combination for a native channel. For example, minK cotransfection with KvLQT1 reproduced the behaviour of the cardiac slow delayed rectifier (Sanguinetti et al. 1996; Barhanin et al. 1996). In the work reported here on mErg1a and KCNQ2+KCNQ3 as candidate channels for the NG108-15 M-like current, the kinetic correspondences are clear, and are supported by pharmacology and molecular biology. Any influence of post-translational modification or accessory subunits is minimal, or is paralleled in the CHO cell line used to express the cloned channels. It seems there is a simple reason for Kv1.2 not looking like an M-current: it isn’t one.

Potassium channel modulation involves multiple steps. The most direct known mechanism involves an agonist acting on a receptor, the receptor activating a G-protein, and the G-protein beta-gamma complex binding to the channel to alter its activity, as happens with cardiac inward-rectifying channels. In the case of M-currents, there is evidence for a
diffusible messenger acting on the channel at a substantial distance from the receptor (Selyanko et al. 1992), implying a fourth or further stage. Individually, these interactions are transient and may be subject to desensitisation, making it difficult to dissect the mechanism of modulation and prove that the mechanism for a heterologously-expressed channel duplicates that for a native counterpart. There is also no guarantee that all the machinery of neuronal transduction will be available in a non-neuronal cell line used to express the receptor and the channel.

Blocker pharmacology is a well-established and valuable tool. Small organic molecules like tetraethylammonium and 4-aminopyridine have long been used for a loose classification of potassium channels. Apamin (for calcium-activated potassium channels) and dendrotoxin (for the Kv1 subfamily) tightened up the standard of current-matching between different cells. It became apparent early on that sensitivity to block by agents like dendrotoxin and tetraethylammonium correlated with the amino-acid sequence of the channel protein (Stühmer et al. 1989; MacKinnon & Yellen 1990; Kavanaugh et al. 1991). The demands of medicine identified blockers such as linopirdine and WAY-123,398 by their effects in specific tissues, before the molecular nature of their targets was known. Molecular biology is now playing an increasing role in channel identification, but blockers remain useful for dissecting mixed currents and for studying function.

It is possible to exploit the direct interaction of a blocker molecule and a potassium channel protein in several ways. A labelled blocker can be used to stain tissue sections, or the blocker can be bound to a chromatographic column to extract the channel from the tissue (e.g. Scott et al. 1990). Electrophysiology monitors the interaction by measuring the blocker's obstruction of the electrical current created by potassium ions flowing through the channel.

There are few theoretical objections to the use of blockers to identify channels. Glycosylation of the extra-membrane region of the channel protein, occurring in some cell types but not others, might conceivably interfere with drug access to a channel mouth. Heteromultimers of two or more types of channel subunit typically show reduced sensitivity to a blocker specific to one of the subunits (as with TEA block of KCNQ2+ KCNQ3 heteromultimers). However, only false negative results in channel matching are likely consequences of these circumstances. The creation of a blocker binding site by heteromultimerisation is possible, but as yet unreported.
Selectivity of drugs

Phylogenetic trees depicting relatedness of potassium channel genes (e.g. Coetzee et al. 1999; Derst & Karschin 1998) emphasise the distinctness of the eag, erg and elk channels. The Shaker-family channels, Kv1, etc., cluster with the KCNQ channels, while the eag group appear on a separate branch which diverged farther back in evolutionary time. The genetic isolation of the ether-à-go-go group of channels might lead one to expect that they would have a distinctive set of blockers (as well as other behaviours) not shared with channels of other families. This was, implicitly, one of the justifications for Stansfeld et al.’s (1997) proposal that the eag family might harbour the channels responsible for the M-current. As it turns out, the KCNQ channels underlying the sympathetic M-current are closely related genetically to a previously-known group. Indeed, for Coetzee et al. (1999): “the question may arise whether the KQT family should be considered as part of the Kv family.” Yet the pharmacological profile of the sympathetic M-current did not betray its closeness to the Kv group.

Are there any grounds for the assumption that binding sites for blockers are conserved within channel families? It depends partly on the nature of the blocker binding site, as I shall explain. For this purpose, it is easier to classify types of blocker (which are smaller molecules) than types of channel.

A number of channel-blocking peptides result from the evolution of animals that make venoms to immobilise prey and defend themselves against predators. They are large molecules, often with high specificity. Alpha-dendrotoxin has 59 amino acids (Harvey 1997) and three identified sites of interaction (Hurst et al. 1991) which confine its blocking effect to some members of the Kv1 channel subfamily. Margatoxin, a Kv1.3-specific blocker from scorpion venom, interacts with multiple sites on its target channel (Aiyar et al. 1995). The more distributed the high-affinity interaction sites, the more specific the blocker is likely to be. The opposite situation arises when sensitivity to a blocker depends on the presence of a single amino acid residue in the channel, as with the tyrosine residue controlling tetraethylammonium block, which may differ among channels in the same family (MacKinnon & Yellen 1990; Kavanaugh et al. 1991; Hadley et al. 2000).

The pharmaceutical industry has created many organic molecules with channel-blocking activity. These molecules need to be complex enough to dock into a specific site, and to be capable of legal protection for their inventors under the patent system. Simultaneously, they must be small enough to be easily administered and to remain dissolved in bodily fluids until they reach their site of action. Reconciling these requirements gives most such drugs
molecular weights in the range of 400-500. Examples are WAY-123,398 (molecular weight 488), azimilide (MW 458 as base, 531 as hydrochloride), THA (MW 235), and linopirdine (MW 391.5). Specificity varies among molecules of this class. Thus, I found WAY-123,398 impressively selective for one constituent of the NG108-15 M-like current. By contrast, azimilide blocks both ERG channels and members of the KCNQ family. Linopirdine at low concentrations is (among potassium channels) selective for KCNQ- and M-channels, but it also acts on ionotropic receptors, and it can block other potassium channels at higher doses (Lamas et al. (1997), and my finding of about 50% block of Kv1.2 by 100 μM linopirdine).

Inorganic cations, the smallest channel blockers, may be physiologically relevant but have little diagnostic use. Calcium, magnesium and zinc have all been implicated as endogenous modulators of various ion channels and receptors. Block by barium is almost universal in potassium channels, so I have not focussed on barium inhibition of Kv1.2, mErg1a or KCNQ channels. The substituted cation tetraethylammonium (TEA) is more useful, showing a range of channel-blocking potencies because of the random occurrence of the channel residue that influences its binding. Consequently, it has proved useful for discriminating KCNQ subunits, as well as showing some differential activity on the two constituents of the NG108-15 M-like current.

Depending on the mixture of currents present, a blocker may point directly to a channel identity (WAY-123,398 on ERG) or a very narrow range of channels (dendrotoxin on the Kv1 subfamily). A combination of blockers may be a better fingerprint for any individual channel, for example linopirdine and TEA for KCNQ2. A lack of sensitivity to a particular blocker can eliminate some candidates, but cannot provide a positive identification.

To conclude, there are some good reasons, rooted in biochemistry, why large and medium-sized channel-blocking molecules can help in the identification of native currents. This will continue to be a good approach to such tasks.

Functional relevance of KCNQ and ERG currents

The M-current in sympathetic ganglion cells has an evident physiological function as a neurotransmitter-sensitive brake on action potential generation (Adams et al. 1982). Nerve cells are designed for versatility: their signalling functions may require a burst of action potentials, a single spike, or none at all for several seconds or minutes. The non-inactivating neuronal M-current builds up over several spikes to halt further firing, but can be switched off by muscarinic receptor activity, allowing both phasic and tonic firing by cells expressing M-
currents. This itself indicates that the continual availability of the M-current is not necessary for the moment-to-moment survival of cells expressing it.

It is however likely that potassium flow through KCNQ channels plays an important part in establishing and maintaining functional neuronal circuitry. Homozygous KCNQ2 knockout mice die just after birth, an effect ascribed tentatively to lung dysfunction caused by continuous depolarisation of adrenal chromaffin cells (Watanabe et al. 2000). The KCNQ2 and KCNQ3 mutations reported in humans do not have a dominant-negative effect, and reduce rather than eliminate channel function (Weinreich & Jentsch 2000). The effect – a short-lived neonatal seizure condition – is moderate and has not been reported to involve sympathetic nervous system dysfunction. Developmental plasticity might compensate for the reduced function of these channels. The recent discoveries of not just KCNQ4, but KCNQ5 (Lerche et al. 2000; Schroeder et al. 2000) raise the possibility that the KCNQ channel family contains enough redundancy to diminish the effects of neuronal channel lesions.

A current resembling the KCNQ2+KCNQ3 heteromultimer is commonly found in NG108-15 cells, but some cells appear able to survive without it (Chapter 4, Discussion). While differentiated NG108-15 and similar cells undoubtedly form synaptic connections with each other, fire action potentials and release neurotransmitters, they can undergo little selection pressure with regard to firing behaviour unless they become fatally flooded with calcium. What differentiated NG108-15 cells do show consistently is a current resembling mErg1a, the mouse homologue of the cardiac fast “delayed rectifier”.

In the heart, the rapidly-inactivating ERG current forms part of a cardiac ion channel complement which allows repeated depolarisations over a defined range of frequencies. If the ERG current is missing, failure of the heart muscle to maintain a steady firing rate results in premature death of the organism. The question remains what (if any) the survival function of ERG currents may be in NG108-15 or other cell lines.

Bianchi et al. (1998) propose that ERG-type “inward rectifier” channels, normally only expressed early in cell differentiation, are “frozen” by neoplastic transformation. These channels then afford tumour cells a selective advantage by maintaining a shallow membrane potential that promotes continual cell division. Their effect on membrane potentials is demonstrated by the fact that the cells used by this group can only be induced to show transient currents (e.g. Arcangeli et al. 1995). Such cells cannot respond to moderate depolarisations with a sustained potassium outflow that would restore a negative potential. Conversely, the “classical” so-called anomalous-rectifier currents from the K\textsubscript{ir} family are
characteristic of mature differentiated cells. These channels stay open above and below the potassium equilibrium potential \(E_k\), allowing ion flow to set the membrane potential close to \(E_k\).

**ERG currents and neuronal differentiation**

Healthy neurones are terminally differentiated – that is, they have stable structural and neurochemical properties and no, or very little, capacity for replacement of damaged tissue. In a neural tumour, or neuroblastoma, neuronal cells have regressed by re-acquiring the ability to divide. NG108-15 cells continue to proliferate when cultured in a high-serum medium without chemical interference, but various treatments cause them to adopt an apparently differentiated state. Some researchers use serum starvation (Arcangeli et al. 1995; Pancrazio et al. 1999). Arcangeli et al. (1995) also used retinoic acid to lock the cells in stages G0-G1 of the cell cycle, in effect differentiating them [see Watson et al. (1992), p.370 for a summary of the cell cycle]. Another standard method of differentiating NG108-15 cells is by raising intracellular cAMP. This (via protein kinase A phosphorylation) activates transcription factors which bind at CREs (cAMP-response elements) to promote transcription of genes (Watson et al. 1992, p329), setting off chemical and morphological changes. My observation is that not all chemically treated cells become morphologically differentiated, and some continue to proliferate, while some untreated cells produce miniature M-like currents. Krystosek (1985) too found a probabilistic relationship between treatment and differentiation.

**ERG currents and the cell cycle**

Several proposals have been made for the function of ERG-like currents in cell lines. An electrical role for HERG-like currents in tumour cell proliferation (Bianchi et al. 1998) is referred to above. A volume-controlling effect of potassium channels may also sustain proliferative activity. Block of potassium channels with TEA, 4-aminopyridine and caesium (effective against HERG at 1 mM: Trudeau et al. 1995), like chloride channel block and other volume-changing stimuli, decreased NG108-15 proliferation. (Rouzaire-Dubois & Dubois 1998). ERG currents may additionally be implicated in the morphological aspects of differentiation: neurite extension in NG108-15 cells was inhibited by 5 mM caesium, but not by the delayed-rectifier blockers 4-aminopyridine and TEA (Pancrazio et al. 1999).

Conversely, the cell cycle appears to affect the properties of ERG-type currents in neuroblastoma (hybrid) cells. Arcangeli et al. (1995) found that in differentiated SH-SY5Y (human neuroblastoma) cells the “inward rectifier” set a more negative resting potential than in proliferating cells. “Inward rectifier” current density increased 40-50 hours after the start of
retinoic acid treatment (Arcangeli et al. 1995). Pancrazio et al. (1999) found that differentiated NG108-15 cells had higher HERG-like current density. ERG mRNA also increased on differentiation, and the voltage dependence of the ERG current shifted by about −15 mV. Meyer & Heinemann (1998) found opposite results in SH-SY5Y cells after 2-3 days treatment with retinoic acid, when differentiated cells showed a positive shift in the voltage-dependence of the HERG-like current.

The activation of HERG is shifted positively, by up to 27 mV, by protein kinase A (Kiehn et al. 1998) acting at four consensus phosphorylation sites (Thomas et al. 1999). These sites in HERG, S283, S890, T895 and S1137, are all conserved in mErg1a (London et al. 1997). Thus, PKA phosphorylation may also cause an activation shift in mErg1a. This could explain what is happening during differentiation: increased PKA activity reduces mErg1a current activity, reducing the membrane potential. Decreased potassium outflow promotes an increase in cell volume, adding to the influences inhibiting cell division. A later rebound is caused either by a down-regulation of kinase activity, or by an overwhelming increase in channel expression. The nature of the ERG currents observed in differentiating/differentiated neuroblastoma cells and their hybrids may therefore be highly sensitive to the amount of time elapsed after the application or removal of differentiating agents, explaining the contradictory results above. The effects of protein kinase A and its inhibition on mErg1a and NG108-15 slow currents would be worth exploring.

With the identification of the slow M-like current in NG108-15 (Meves et al. 1999; Selyanko 1999), it has become clear that an ERG current persists in the NG108-15 line long after differentiation. A minimal conclusion accommodating Bianchi et al.’s (1998) argument is that the ERG-type current is permissive with regard to tumour-like behaviour, but has other roles in non-proliferating cells. Given that ERG channel mRNAs are expressed in the mammalian brain (Shi et al. 1997; Wymore et al. 1997), a role in mature neurones seems likely. The lack of neurological correlates of HERG-related long QT syndrome may be explained by the compensatory effect of other family members (Shi et al. 1997).

Stoichiometry, assembly and membrane complexes of KCNQ channels

In my work, I transfected equal amounts of KCNQ2 and KCNQ3 plasmids into GHO cells, hoping that these cells would process the subunits in the same way as NG108-15 cells. GHO cells yielded a presumed heteromultimer with high expression, and intermediate block by TEA, compared with KCNQ2 or KCNQ3 alone. My proposal is that these consistent properties may point to KCNQ2 and KCNQ3 coassembling by preference rather than by random association. If the two subunits have a higher affinity for each other than for their
own kind, the most likely assembly is a tetramer in which they are linked 2-3-2-3, giving a
two-plus-two ratio of subunits. An alternative possibility is that subunits assemble randomly,
but that heteromultimeric channels are more efficiently trafficked to the membrane. Schwake
et al. (2000) showed increased – and quantitatively similar – surface expression of epitope-
tagged KCNQ2 and KCNQ3 subunits co-expressed in oocytes, and suggested an analogy
with ATP-sensitive potassium channels, where coassembly of different subunits conceals a
peptide motif normally responsible for protein retention in the endoplasmic reticulum.

The findings of Shapiro et al. (2000) differ from mine, and from reports on KCNQ channels in
oocytes, in that their HEK cell line expressed KCNQ2 less readily than KCNQ3. Perhaps
HEK cells have different trafficking mechanisms from CHO cells and oocytes. Alternatively,
there may be differences in the interaction of channels with the intracellular scaffolding.
Cooper et al. (2000) demonstrate that human brain KCNQ2+KCNQ3 channels are associated
with a sub-membrane complex of tubulin, actin, protein kinase A and the anchoring protein
AKAP79, with KCNQ2 rather than KCNQ3 containing the region responsible for cytoskeletal
interaction. It will be interesting to see whether human embryonic kidney cells (and other cell
lines) also have a mechanism for pinning KCNQ2 channels to the cell surface.

**Future directions**

Several approaches are under way to corroborate the presence of functional ERG- and
KCNQ-family channels in the NG108-15 line, including staining with anti-KCNQ2 and anti-
KCNQ3 antibodies, and transfection of dominant-negative mutations of KCNQ2 and KCNQ3
(with a G→S pore mutation preventing potassium ion flow) into NG108-15 cells. To confirm
the expression of such channels, an epitope tag such as Myc or FLAG on the mutated
channel subunit enables the use of antibodies that specifically visualise the mutated
channels. A suitably-tagged dominant-negative mutant mErg1a will be needed to apply the
same approach to the NG108-15 slow current.

The single channels so far recorded from NG108-15 cells (Selyanko et al. 1995) appear to
correspond to the KCNQ-like constituent of the current, which matches the classic SCG M-
current. Where are the ERG channels? The single-channel behaviour of mErg1a in a
mammalian cell environment needs to be examined, but the sparseness of mErg1a
expression in transfected CHO cells has been discouraging.

Discrepancies in the reported effect of protein kinase A phosphorylation on the M-current
warrant further investigation. Schroeder et al. (1998) reported a PKA-mediated increase in
KCNQ2+KCNQ3 currents in *Xenopus* oocytes and HEK cells, but other researchers found no effect of membrane-permeable cAMP analogues on the M-current (Brown et al. 1989) or PKA activators on KCNQ2 (Tinel et al. 1998). Work by Kiehn et al. (1998) and Thomas et al. (1999) shows inhibition of HERG by PKA acting at sites conserved in mErg1a. In NG108-15 cells at least, kinase effects may have been missed in the past because of opposite effects on the two channel types contributing to the M-like current. Combining selective block of one component with PKA activation is now a possibility.

Taking a broader view, M-currents in autonomic neurones and M-like currents in their tumour-cell relatives are only the tip of the iceberg. The new frontier is, of course, the brain. Apart from the hippocampal M-current, M-like currents in brain neurones have been little studied as yet. Noda et al. (1998) found an M-like current in rat cortical pyramidal neurones which had a relatively high linopirdine IC$_{50}$ of 35.8 µM, giving grounds to suspect that an ERG or other linopirdine-insensitive channel type was present. With our present knowledge of blockers such as WAY-123,398, pharmacological dissection of brain M-like currents, as they emerge, should be a fruitful approach.
References


conserved in tumors of different histogenesis: a selective advantage for cancer cells? 


Bleich, M, Briel, M, Busch, AE, Lang, HJ, Gerlach, U, Gögelein, H, Greger, R & Kunzelmann, K (1997). K<sup>⁺</sup>LQT channels are inhibited by the K<sup>⁺</sup> channel blocker 293B. *Pflügers Arch.* **434**, 499-501.


sensitivity to neurotoxins, and regulation of the brain delayed-rectifier $K^+$ channel Kv1.2. 


Rapid Report

Inhibition of KCNQ1-4 potassium channels expressed in mammalian cells via M1 muscarinic acetylcholine receptors

A. A. Selyanko, J. K. Hadley, I. C. Wood*, F. C. Abogadie*, T. J. Jentsch† and D. A. Brown

Department of Pharmacology, *Wellcome Laboratory for Molecular Pharmacology, University College London, Gower Street, London WC1E 6BT, UK and †Zentrum für Molekulare Neurobiologie Hamburg (ZMNH), Universität Hamburg, Martinistrasse 85, D-20246 Hamburg, Germany

(Received 22 October 1999; accepted after revision 6 December 1999)

1. KCNQ1-4 potassium channels were expressed in mammalian Chinese hamster ovary (CHO) cells stably transfected with M1 muscarinic acetylcholine receptors and currents were recorded using the whole-cell perforated patch technique and cell-attached patch recording.

2. Stimulation of M1 receptors by 10 μM oxotremorine-M (Oxo-M) strongly reduced (to 0-10%) currents produced by KCNQ1-4 subunits expressed individually and also those produced by KCNQ2+KCNQ3 and KCNQ1+KCNQ2 heteromers, which are thought to generate neuronal M-currents (I\textsubscript{M}) and cardiac slow delayed rectifier currents (I\textsubscript{Ks}), respectively.

3. The activity of KCNQ2+KCNQ3, KCNQ2 and KCNQ3 channels recorded with cell-attached pipettes was strongly and reversibly reduced by Oxo-M applied to the extra-patch membrane.

4. It is concluded that M1 receptors couple to all known KCNQ subunits and that inhibition of KCNQ2+KCNQ3 channels, like that of native M-channels, requires a diffusible second messenger.

KCNQ1-4 are potassium channels abundantly expressed in various tissues. KCNQ1 has a high level of expression in the heart where it co-assembles with minK (KCNEl) channels to give a slow delayed rectifier type current (I\textsubscript{Ks}; Barhanin et al. 1996; Sanguinetti et al. 1996; Yang et al. 1997). Mutations in KCNQ1 contribute to the long QT syndrome which causes cardiac arrhythmia and sudden death (Sanguinetti, 1999). KCNQ2 and KCNQ3 are expressed exclusively in the nervous system. Mutations in either KCNQ2 or KCNQ3 result in a juvenile epilepsy (Biervert et al. 1998; Charlier et al. 1998; Singh et al. 1998; Lerche et al. 1999). KCNQ4 is expressed in cochlea and brain and its mutations cause deafness (Kubiach et al. 1999).

Heteromeric KCNQ2+KCNQ3 channels probably constitute the M-channel in sympathetic ganglia (Wang et al. 1998). M-channels control neuronal excitability (Brown, 1988; Marrion, 1997) and can be inhibited via M1 muscarinic acetylcholine receptors (Marrion et al. 1998; Bernheim et al. 1992; Hamilton et al. 1997) to produce a slow cholinergic excitation. When expressed in Xenopus oocytes, heteromeric KCNQ2+KCNQ3 channels can also be inhibited through M1 receptors (Wang et al. 1998). In the Xenopus expression system KCNQ4 produces a potassium current similar to the M-current (Kubiach et al. 1999) and a native analogue of the KCNQ4 current has recently been identified in mouse outer hair cells (Marcotti & Kros, 1999). However, sensitivity of KCNQ4 currents to muscarinic receptor stimulation is unknown.

The present experiments were aimed at establishing whether the muscarinic inhibition of KCNQ2+KCNQ3 channels (i) involves one of these subunits or both; (ii) is membrane-delimited or requires a diffusible second messenger; and (iii) can be extended to KCNQ1 and KCNQ4 subunits which have shorter carboxy termini and, therefore, a reduced number of potential regulatory sites.

METHODS

Cell culture and transfection

The procedure for Chinese hamster ovary (CHO) hm1 cell culture and transfection has been described previously (Selyanko et al. 1999). Briefly, CHO hm1 cells, expressing the human M1 receptor (Mullaney et al. 1995), were grown at 37 °C and 5% CO\textsubscript{2} in alpha-modified Eagle’s medium (MEM) supplemented with 10% fetal calf serum, 1% L-glutamine and 1% penicillin–streptomycin. Cells plated in 35 mm plastic dishes were transfected 1–2 days after plating using ‘LipofectAmine Plus’ (Gibco BRL). Plasmids containing KCNQ and CD8 cDNAs, driven by CMV promoter, were co-transfected in a channel to marker ratio of 10:1. For
heteromultimers equal amounts of KCNQ2 and KCNQ3 cDNAs were used. Cells expressing channels were identified by adding CD8-binding Dynabeads (Dynal A/S) before recording.

Whole-cell perforated patch recording

Recordings were made at room temperature (20–22 °C) 1–4 days after transfection (longer times were required to obtain sufficiently large KCNQ2 and KCNQ3 currents). Cells were bath perfused with the following solution (mM): 144 NaCl, 2-5 KCl, 2 CaCl2, 0-5 MgCl2, 5 Hepes, 10 glucose, pH 7-4 with Tris base. Pipettes were filled with an internal solution containing (mM): 80 potassium acetate, 30 KCl, 40 Hepes, 3 EGTA, 1 CaCl2, pH 7-4 with KOH. The liquid junction potential was small (4 mV; Selyanko et al. 1995) and so no correction was made. Amphotericin B was used to perforate the patch (Rae et al. 1991). Perforated patch recording leads to an additional undischarged Donnan potential due to the impermeability of amphotericin pores to large intracellular anions. Assuming all intrapipette anions are permeant through the pores and all intracellular anions impermeant, the Donnan potential would be ≤ 11 mV (see Horn & Marty, 1988). Pipette resistance was 2–3 MΩ and the series resistance was compensated (60–90%).

Cell-attached single-channel recording

Single-channel recordings were made 2–4 days after transfection, when the density of channels was enhanced. The methods of recording and analysis were similar to those previously employed for studying the M-channels (Selyanko & Brown, 1999). Intrapipette solution had the same composition as the bath solution used for whole-cell recording. In cell-attached experiments bath solutions had elevated (25 mM) K+ to pre-depolarise the cell at ~−30 mV. Records were sampled at 4-46 kHz and filtered at 0.5–1 kHz. Pipette resistance was 2–3 MΩ.

In patches containing single channels, the effects of muscarinic stimulation on single-channel activity were deduced from changes

Figure 1. Activation (left panel, onsets in boxes) and deactivation (right panel) of homomeric (KCNQ1-4) and heteromeric (KCNQ2+KCNQ3) channels expressed in CHO cells

Currents were activated by long (1–2 s; 0.06 Hz) depolarising pulses from a holding potential of −70 mV, and deactivated by 1–2 s hyperpolarising pulses from the full activation potential of +50 mV (see top records for the voltage protocols).
in the open probability while the multi-channel activity was measured as the mean current through these channels.

**Data acquisition and analysis**

Data were acquired and analysed using pCLAMP software (version 6.0). Currents were recorded using an Axopatch 200A (or 200B) patch-clamp amplifier, filtered at 1 kHz and digitised at 4–8 kHz. Activation curves were fitted by the Boltzmann equation:

\[
\frac{I/I(50)}{1 + \exp(V_m - V_{50})/k},
\]

where \( I \) is the tail current recorded at \(-70\) mV following a step to membrane potential \( V_m \), \( I(50) \) is current following a step to \(+50\) mV, and \( V_{50} \) is the membrane potential at which \( I \) is equal to \( 0.5I(50) \). Current size and its inhibition by a muscarinic stimulant was measured from the amplitude of the deactivation tail recorded at \(-50\) mV. The program 'Origin' (version 5.0, Microcal Software Inc.) was used for fitting activation curves and for creating the figures. Data are shown as means ± s.e.m. unless otherwise stated.

**Drugs and chemicals**

cDNAs for KCNQ1, KCNQ2, KCNQ3 and KCNQ4 were human and KCNQ3 cDNA was from the rat. Oxo-M (oxotremorine methiodide) was obtained from RBI (Natick, MA, USA). All other drugs and chemicals were obtained from Sigma or BDH (Poole, UK).

**RESULTS**

**Activation of KCNQ1-4 channels**

Figure 1 shows examples of currents produced by different KCNQ subunits expressed in CHO cells. All currents had low thresholds (–60 mV), slow and sustained activations and slow deactivations. Activation curves for homomeric (KCNQ1, KCNQ2, KCNQ3, KCNQ4) and heteromeric (KCNQ2+KCNQ3) channels (Fig. 2) were fitted by the Boltzmann equation at \( V_{50} \) and \( k \), respectively, equal to

![Figure 2. Activation curves for KCNQ1-4 and KCNQ2+KCNQ3 channels](image)
-7.8 ± 1.2 mV and 10.0 ± 1.1 mV, KCNQ1 (number of cells, n = 6); -13.8 ± 0.4 mV and 12.1 ± 0.4 mV, KCNQ2 (n = 5); 36.8 ± 1.3 mV and 5.5 ± 1.1 mV, KCNQ3 (n = 3); -18.6 ± 0.3 mV and 9.8 ± 0.2 mV, KCNQ4 (n = 7); and -17.7 ± 0.6 mV and 11.9 ± 0.5 mV, KCNQ2+KCNQ3 (n = 6). Previously reported half-activation voltages for KCNQ1 (-2.4 mV, Chouabe et al. 1997), KCNQ2 (-3.7 mV, Biervert et al. 1998), KCNQ4 (-1.0 mV, Kubisch et al. 1999) and KCNQ2+KCNQ3 (-40 mV, Wang et al. 1998) channels differed somewhat from those obtained in the present experiments. The uncorrected junction potential of ~4 mV and (uncertain) Donnan potential in our perforated-patch recording (see Methods) might contribute to some of the more positive half-activation voltages we have obtained, but this would not explain all the differences. These may therefore be associated with the different expression systems used.

The current onsets showed sigmoidal activation (in KCNQ4 only at very positive potentials) and all except KCNQ4 channels showed evidence of some inactivation at positive membrane potentials: they either slowly declined during continuous membrane depolarisation (KCNQ2 and KCNQ3) or 'saturated' with increased depolarization (KCNQ1 and KCNQ2+KCNQ3). Hyperpolarising voltage steps from the full activation potential of +50 mV removed inactivation and increased the currents.

M₄ receptor inhibition of KCNQ1–4 channels
Figure 3.4 shows that stimulation of M₄ receptors by oxotremorine-M (Oxo-M) strongly reduced the KCNQ2+KCNQ3 current (on average, to 11.6 ± 11.4%, n = 3). When recorded in cell-attached patches, the activity of KCNQ2+KCNQ3 channels was reduced by adding Oxo-M to the bath (see Fig. 3B; average inhibition to 6.4 ± 2.8%; 8 patches).

Figure 4 shows that KCNQ1–4 subunits expressed individually can also be inhibited by Oxo-M, to 3.0 ± 2.9% (n = 5), 9.8 ± 1.1% (n = 3), 0.0 ± 0.0% (n = 6) and 3.9 ± 3.9% (n = 5), in subunits 1–4, respectively. When recorded in cell-attached patches, the activities of KCNQ2 and KCNQ3 channels were inhibited by Oxo-M added to the bath, to 2.2 ± 2.0% (5 patches) and 5.3 ± 4.5% (3 patches), respectively (not shown).

Compared with homomeric KCNQ1 channels, heteromeric (‘cardiac’) KCNQ1+KCNE1 channels produced larger currents which had a lower threshold and slower activation kinetics (see also Barhanin et al. 1996; Sanguinetti et al. 1996; Yang et al. 1997). These KCNQ1+KCNE1 currents were also abolished by Oxo-M (n = 3; see Fig. 4).

All effects of Oxo-M on KCNQ1–4 channels were sustained and slowly reversible.
DISCUSSION

The idea that KCNQ2+KCNQ3 channels form M-channels is based on the overlapping distributions of KCNQ2-, KCNQ3- and M-channels in the nervous system and the similarities of the biophysical and pharmacological properties of their macroscopic currents (Wang et al. 1998). The present experiments give additional new support to this idea. Thus, KCNQ2+KCNQ3 channels were inhibited via activation of M1 receptors (as previously reported in oocytes by Wang et al. 1998); and furthermore, cell-attached recording showed that, like native M-channels (Selyanko et al. 1992), this inhibition required a diffusible second messenger. Since in CHO cells M1 receptors couple to the G protein Gq (Mullaney et al. 1993), M1-induced inhibition of KCNQ2+KCNQ3 channels, like that of native ganglionic M-channels (Haley et al. 1998), may be mediated by the αq subunit. Interestingly, average inhibition of KCNQ2+KCNQ3 currents by 10 μM Oxo-M (88%, present experiments) was not different from that for the M-currents in mammalian sympathetic neurones (~90%; Hamilton et al. 1997; Haley et al. 1998).

The present experiments showed that homomeric KCNQ2 and KCNQ3 channels can also be inhibited via activation of M1 muscarinic receptors, and also through a diffusible second messenger. This suggests that both subunits forming the M-channel couple to M1 muscarinic receptors, probably through the same mechanism.

Two other members of the subfamily, KCNQ1 and KCNQ4, have not previously been considered as contributing to M-channels largely because one of them, KCNQ1, is seen as a ‘cardiac’ channel, with low expression in the brain (Yang et al. 1997). The other, KCNQ4, has not yet been extensively studied, and its distribution in the brain remains to be determined (Kubisch et al. 1999). The results obtained in the present experiments with expressed KCNQ1 and KCNQ4

![Figure 4. Inhibition of KCNQ1-4 channels via stimulation of M1 muscarinic acetylcholine receptors](image-url)

Experimental protocol as in Fig. 3.4. Responses on recovery were recorded 10–15 min after washing out Oxo-M.
channels are interesting in three respects. First, when recorded with the standard deactivation M-current protocol, KCNQ1 and KCNQ4 currents had kinetics strikingly similar to those of the M-current, and these channels too were inhibited via stimulation of M, muscarinic receptors. Thus, all members of the KCNQ family reported so far can generate 'M-channels' as defined kinetically and pharmacologically.

Second, acetylcholine is a transmitter in both the heart and the inner ear, so if M receptors and corresponding KCNQ subunits are co-expressed in the same cells, their coupling may influence cardiac function and hearing. (Acetylcholine has been reported to reduce $I_{Ks}$ when enhanced by isoproterenol (Harvey & Hume, 1983; Yazawa & Kemeysama, 1989), but this was probably due to inhibition of adenylate cyclase, and so was probably mediated by $M_2$ rather than $M_1$ receptors. Also, Freeman & Kass (1995) reported that $I_{Ks}$ in guinea-pig sino-atrial node cells was inhibited by cholinergic agonists but via a direct interaction with $I_{Ks}$ channels.)

Third, because KCNQ1 and KCNQ4 subunits have short carboxy termini (compared with those of KCNQ2+KCNQ3 subunits; see Wang et al. 1998; Kubisch et al. 1999) this allows us to eliminate substantial parts of KCNQ2+KCNQ3 C-termini as obligatory sites involved in channel inhibition.

Thus, although the mechanism of muscarinic inhibition of M- (and KCNQ-) channels is still unknown, it is clearly conserved among all four members of the KCNQ family.

---


**Acknowledgements**

This work was supported by the MRC and the Wellcome Trust. We thank Dr D. McKinnon for KCNQ2 and KCNQ3 cDNAs and Dr M. T. Keating for KCNQ1 cDNA.

**Corresponding author**

A. A. Selyanko: Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, UK.

Email: a.selyanko@ucl.ac.uk

**Author's present address**

I. C. Wood: School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK.
SPECIAL REPORT

Differential tetraethylammonium sensitivity of KCNQ1–4 potassium channels

J.K. Hadley, M. Noda, A.A. Selyanko, I.C. Wood, F.C. Abogadie & D.A. Brown

Introduction

The KCNQ family of channels includes the cardiac slow delayed rectifier KCNQ1 (KvLQT1) (Barhanin et al., 1996; Sanguineti et al., 1996), the neural-specific channels KCNQ2 and KCNQ3 (Biervert et al., 1998; Charlier et al., 1998; Yang et al., 1998), which have been proposed as components of the M-channel (Wang et al., 1998), and KCNQ4, whose mRNA is expressed in the cochlea (Kubisch et al., 1999). The TEA sensitivity of KCNQ2 is higher than that of the M-channel, that of KCNQ3 is lower, and that of the KCNQ2/KCNQ3 heteromultimer closely matches that of the M-channel, that of KCNQ3 is lower, and that of the KCNQ2/KCNQ3(T323Y), 0.5. While the high TEA sensitivity of KCNQ2 may be conferred by a tyrosine residue lacking in the other channels, the intermediate TEA sensitivity of KCNQ1 and KCNQ4 implies that other residues are also important in determining TEA block of the KCNQ channels.

Methods

Methods for culture and transfection of CHO hml cells have been described previously in Selyanko et al. (1999). CHO cells (a derivative of the CHO-K1 line; Mullaney et al., 1993) were grown at 37°C and 5% CO₂ in α-MEM supplemented with 10% foetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin. For recording, cells were plated in 35 mm plastic dishes, and transfected 1–2 days after plating using Lipofect Amine Plus (Life Technologies) as suggested by the manufacturer. Plasmids (driven by CMV promoter) containing cDNAs for KCNQ channels, and cDNA for CD8 as a marker, were cotransfected at a channel : marker ratio of 10:1. Cells for patch-clamping were identified using CD8-binding Dynabeads (Dynal U.K.) Ltd 1 day after transfection.

The perfusing solution contained (mM): NaCl 144, KCl 2.5, CaCl₂ 2, MgCl₂ 0.5, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) 5, and glucose 10 plus Tris base to pH 7.4. The pipette solution contained (mM): K acetate 80, KCl 30, HEPES 40, MgCl₂ 3, ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA) 3, CaCl₂ 1 and NaOH to pH 7.4. Amphotericin B was used to perforate the patch (Rae et al., 1991). TEA was applied cumulatively, using 0.2 m and 2 m aqueous TEA solutions added to the reservoir of perfusing fluid.

Data were acquired and analysed using pClamp software (version 6.0.3). Currents were recorded using an Axopatch 200A (or 200) patch-clamp amplifier, filtered at 1 kHz, and digitized at 1–4 kHz. One second steps to −50 mV were applied from a holding potential of −20 mV and the total deactivation relaxation amplitude was measured at −50 mV in control and for each dose of TEA. Data were normalized and plotted in Origin (version 5.0, Microcal Software), and curves for each group were fit to the Hill equation using Origin (version 5.0, Microcal Software).

Abbreviations: CHO (hml), Chinese hamster ovary cell (stably expressing human M, muscarinic receptors); EGTA, ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; IC₅₀, drug concentration giving 50% block of current; M-channel/M-current, muscarinic-inhibited potassium channel/current; NG108-15, neuroblastoma-glioma hybrid cell line; nH, Hill slope; pIC₅₀, negative log of IC₅₀; TEA, tetraethylammonium

Keywords: TEA; KvLQT1; KCNQ channels; M-current; Chinese hamster ovary (CHO) cell
were fitted with the equation: $y = \frac{1}{1 + (x/x_0)^p}$ where $y$ is the relative current amplitude; $x$ is the concentration of the blocker; $x_0$, the IC$_{50}$, is the concentration at which $y$ is 0.5; and $p$ is the power (equivalent to the Hill slope, $n_H$). Additionally, data were plotted against log concentrations and fitted with the Boltzman equation: $y = \frac{1}{1 + e^{-(x-x_0)/a}}$, where $x_0$ is the pIC$_{50}$ (negative log of the IC$_{50}$), as a check on the true (geometric) spread of the standard error.

TEA was from Lancaster Synthesis. All other chemicals were obtained from Sigma or BDH Chemicals. Human KCNQ1 cDNA was kindly provided by Dr M.T. Keating; human KCNQ2 and rat KCNQ3 (as studied in Wang et al., 1998) by Dr D. McKinnon; and human KCNQ4 (as in Kubisch et al., 1999) by Dr T.J. Jentsch. We thank Dr T.J. Jentsch and Tatjana Kharkovets for the mutated KCNQ3(T323Y), which was based on the human sequence.

Results We find that the TEA sensitivity of KCNQ2 is high, that of KCNQ3 is low, and that of KCNQ1 and KCNQ4 is intermediate. Figure 1Aa - d shows representative data records for TEA inhibition of homomeric KCNQ channel currents. Figure 2A and Table 1 show the potencies for these channels. Figure 1Ba,b shows data records for the heteromeric channels produced by co-transfecting KCNQ2 with wild-type KCNQ3 or with the mutated KCNQ3 subunit KCNQ3(T323Y). As is clear from Figure 2B and Table 1, the potency for the heteromeric wild-type KCNQ2 and KCNQ3 was intermediate between those of homomeric KCNQ2 and KCNQ3. The potency for heteromers containing the mutated KCNQ3(T323Y) was increased, approaching that for the wild-type KCNQ2.

Discussion Our results for TEA inhibition of KCNQ2 and KCNQ2/3 currents in CHO cells give IC$_{50}$ values of $0.3 \pm 0.02$ mM and $3.8 \pm 0.2$ mM (pIC$_{50}$ values $3.53 \pm 0.03$ and $2.42 \pm 0.02$), respectively. These values accord well with previously reported $K_a$ values for these channels expressed in Xenopus oocytes, which were $0.16 \pm 0.02$ mM and $3.5 \pm 0.7$ mM, respectively (Wang et al., 1998). The sensitivity of KCNQ3 to TEA blockade has not been quantified, but 5 mM TEA had a negligible effect on KCNQ3 currents in oocytes (Yang et al., 1998). Even the highest concentration of TEA tested in the present experiments, 30 mM, which could completely block KCNQ1, 2 and 4, had a minimal effect on KCNQ3 (Figure 2A).

TEA block does not appear to have been tested before on KCNQ1 and KCNQ4 channels, so it was of interest to compare TEA sensitivity of these channels with that of the

Table 1 Half-maximal blocking concentrations of TEA (IC$_{50}$) in mM, pIC$_{50}$ (negative log of the IC$_{50}$ in mM, fitted separately), and Hill slopes ($n_H$) for block of KCNQ channels expressed in CHO cells; $n$ = number of cells tested

<table>
<thead>
<tr>
<th>Channel</th>
<th>IC$_{50}$ ± s.e.mean (mM)</th>
<th>pIC$_{50}$ ± s.e.mean</th>
<th>$n_H$ ± s.e.mean</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNQ1</td>
<td>5.0 ± 0.2</td>
<td>2.30 ± 0.02</td>
<td>1.8 ± 0.1</td>
<td>4</td>
</tr>
<tr>
<td>KCNQ2</td>
<td>0.3 ± 0.02</td>
<td>3.53 ± 0.03</td>
<td>0.9 ± 0.06</td>
<td>4</td>
</tr>
<tr>
<td>KCNQ3</td>
<td>&gt;30</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>KCNQ4</td>
<td>3.0 ± 0.3</td>
<td>2.53 ± 0.04</td>
<td>2.0 ± 0.4</td>
<td>3</td>
</tr>
<tr>
<td>KCNQ2,3</td>
<td>3.8 ± 0.2</td>
<td>2.42 ± 0.02</td>
<td>0.8 ± 0.03</td>
<td>4</td>
</tr>
<tr>
<td>(T323Y)</td>
<td>0.5 ± 0.05</td>
<td>3.29 ± 0.05</td>
<td>1.0 ± 0.09</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 1 Differential TEA sensitivities of KCNQ1 – 4 (Aa – d) and heteromeric KCNQ2/KCNQ3 and KCNQ2/KCNQ3(T323Y) currents (Ba,b), expressed in CHO cells. Deactivations were recorded in response to 1 s steps from the holding level of $-20$ to $-50$ mV, in the absence and presence of different concentrations of TEA as indicated (in mM) on the left side of each trace.
Figure 2 Concentration dependences of inhibition of KCNQ1-4 currents (A), and coexpressed KCNQ2/KCNQ3 and KCNQ2/KCNQ3(T323Y) channels (B) in CHO cells. All values are mean ± s.e.mean. Smooth lines are least squares fits to the equation $y = \frac{l}{1 + (x/x_0)^n}$, superimposed for all data sets except KCNQ3, where the Hill coefficient of 0.8 for TEA inhibition of KCNQ2/3 heteromers matches the previously reported value of 0.8 for these channels (Yang et al., 1998), and is comparable with values of 0.6 for both the mouse superior cervical ganglion M-current and the fast component of the neuroblastoma-glioma hybrid (NG108-15) M-like current (Selyanko et al., 1999).

We find that substituting tyrosine for threonine at position 323 in KCNQ3 apparently increases the TEA sensitivity of the heteromeric KCNQ2/3 channel, as predicted from previous work on Shaker channels. This implies that homomeric KCNQ3(T323Y) channels should also be considerably more sensitive to TEA than wild-type KCNQ3 channels, but unfortunately we were unable to obtain homomeric KCNQ3(T323Y) currents of sufficient amplitude to test their TEA sensitivity directly. However, the fact that KCNQ1 and KCNQ4, lacking a tyrosine in this position, also have a higher TEA sensitivity than KCNQ3, suggests that other residues are important in determining TEA block of KCNQ channels.

Finally, it is worth noting that both KCNQ1 and KCNQ4 yield currents with a similar appearance to M-currents (Figure 1). Since their sensitivity to TEA (and to the M-channel blocker, linopirdine: Kubisch et al., 1999; A.A. Selyanko, unpublished observations) is similar to that of native M-currents, these may also be regarded as representing species of 'M-channels'. The characteristics of TEA blockade described here may be useful in establishing whether KCNQ1 and KCNQ4 participate in the formation of any native M-like channels.

This work was supported by the M.R.C. and The Wellcome Trust. We thank Dr T.J. Jentsch for helpful reading of the manuscript.

References


(Received October 21, 1999
Revised November 1999
Accepted November 10, 1999)
Two Types of $K^+$ Channel Subunit, Erg1 and KCNQ2/3, Contribute to the M-Like Current in a Mammalian Neuronal Cell


1Department of Pharmacology, 2Wellcome Laboratory for Molecular Pharmacology, University College London, London, WC1E 6BT, United Kingdom, and 3Cardiovascular Institute, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15213

The potassium M current was originally identified in sympathetic ganglion cells, and analogous currents have been reported in some central neurons and also in some neural cell lines. It has recently been suggested that the M channel in sympathetic neurons comprises a heteromultimer of KCNQ2 and KCNQ3 (Wang et al., 1998) but it is unclear whether all other M-like currents are generated by these channels. Here we report that the M-like current previously described in NG108–15 mouse neuroblastoma x rat glioma cells has two components, “fast” and “slow”, that may be differentiated kinetically and pharmacologically. We provide evidence from PCR analysis and expression studies to indicate that these two components are mediated by two distinct molecular species of $K^+$ channel: the fast component resembles that in sympathetic ganglia and is probably carried by KCNQ2/3 channels, whereas the slow component appears to be carried by merg1a channels. Thus, the channels generating M-like currents in different cells may be heterogeneous in molecular composition.

Key words: potassium channels; neuroblastoma x glioma hybrid cells; M current; sympathetic neuron; Erg1; KCNQ

The M current ($I_{K(M)}$) is a low-threshold, slowly activating potassium current that exerts an inhibitory control over neuronal excitability; this inhibition can be relieved by neurotransmitters acting on G-protein-coupled receptors, leading to enhanced excitability and reduced spike-frequency adaptation (Brown, 1988; Marrion, 1997). The current was originally described in sympathetic neurons (Brown and Adams, 1980; Constanti and Brown, 1981), and analogous currents have subsequently been identified in a variety of other neuronal and non-neuronal cells. Because the precise kinetic and pharmacological properties of the current vary somewhat in different cell types, the name “M-like” is often applied to this current family.

Recently, evidence has been provided to indicate that the channels that generate the M current in rat sympathetic neurons are composed of a heteromultimer of KCNQ2 and KCNQ3 subunits (Wang et al., 1998; see also Yang et al., 1998). These are the two homologs of the KCNQ1 (KvLQT1) channel, mutations of which are responsible for one form of the cardiac “long QT” syndrome (Yang et al., 1997). In contrast, KCNQ2 and KCNQ3 are restricted to the nervous system, and mutations in these channels are associated with a form of infant epilepsy termed “benign familial neonatal convulsions” (Biervert et al., 1998; Charlier et al., 1998; Schroeder et al., 1998; Singh et al., 1998). However, it is not yet known whether all M-like channels are composed of these two subunits (or homologs thereof), or whether members of other $K^+$ channel gene families might contribute to the generation of M-like currents.

In the present experiments, we have attempted to identify the molecular species of $K^+$ channels that generate the M-like current ($I_{K(M,ng)}$) in NG108–15 mouse neuroblastoma x rat glioma cells. These currents have been particularly well characterized (Higashida and Brown, 1986; Brown and Higashida, 1988a,b; Fukuda et al., 1988; Schafer et al., 1991; Robbins et al., 1992, 1993; Selyanko et al., 1995). Like the channels in sympathetic neurons, they are inhibited by transmitters acting on G-protein-linked receptors coupled to phospholipase C (e.g., bradykinin and M1 and M3 muscarinic receptors) (Higashida and Brown, 1986; Fukuda et al., 1988), with similar consequences for cell firing (Robbins et al., 1993). On the other hand, the kinetics of $I_{K(M,ng)}$ appear more complex than those of the ganglionic M current (Robbins et al., 1992), and the two currents differ in their sensitivities to 9-aminotetrahydroacridine (cf. Marsh et al., 1990; Robbins et al., 1992) and linopirdine (cf. Aiken et al., 1995; Lamas et al., 1997; Noda et al., 1998). It has previously been suggested that Shaker-type Kv1.2 channels, cloned from NG108–15 cells (Yokoyama et al., 1989), may contribute to $I_{K(M,ng)}$ (Morielli and Peralta, 1995). However, the insensitivity of $I_{K(M,ng)}$ to dendrotoxin (Selyanko et al., 1995) makes this unlikely. Instead, we provide evidence to indicate that two different types of $K^+$ channel contribute to the M-like current in NG108–15 cells: the mouse other-a-go-go-related gene (merg1a), also expressed in the brain (London et al., 1997), and KCNQ2/KCNQ3, the proposed substrate for the ganglionic current (Wang et al., 1998).

MATERIALS AND METHODS

Cell cultures. NG108–15 mouse neuroblastoma x rat glioma hybrid cells, subclone BM8 (PM1), transfected to express pig brain M1 muscarinic receptor (Fukuda et al., 1988), were cultured and differentiated as described previously (Robbins et al., 1992). Chinese hamster ovary (CHO) cells stably transfected with cDNA encoding human M1 muscarinic
Table 1. Fast and slow kinetic components of $I_{K_{M,ng}}$ and their comparison with the kinetics of $I_{K_{M,ne}}$ and $I_{Merga}$

<table>
<thead>
<tr>
<th>Current</th>
<th>Fast contribution</th>
<th>Slow contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I_{K_{M,ng}}$ Total</td>
<td>117 ± 5 msec</td>
<td>658 ± 29 msec</td>
</tr>
<tr>
<td></td>
<td>33.1 ± 2.1% (86/101)</td>
<td>25.7 ± 1.7% (82/101)</td>
</tr>
<tr>
<td></td>
<td>33 ± 126 msec</td>
<td>2356 ± 247 msec</td>
</tr>
<tr>
<td>Fast (WAY-insensitive)</td>
<td>104 ± 7 msec (37)</td>
<td>456 ± 29 msec</td>
</tr>
<tr>
<td>Slow (WAY-sensitive)</td>
<td></td>
<td>41.7 ± 3.5% (34/36)</td>
</tr>
<tr>
<td>$I_{K_{M,ne}}$</td>
<td>93 ± 5.6 msec (n = 15)</td>
<td>183 ± 10.6 msec</td>
</tr>
<tr>
<td>$I_{Merga}$</td>
<td>64.6 ± 2.4% (22/23)</td>
<td>35.4 ± 2.4% (23/23)</td>
</tr>
<tr>
<td>$I_{Mergib}$</td>
<td>37.1 ± 19.4 msec</td>
<td>112 ± 42.5 msec</td>
</tr>
<tr>
<td></td>
<td>70.8 ± 15.8% (3/3)</td>
<td>29.2 ± 15.8% (3/3)</td>
</tr>
</tbody>
</table>

$I_{K_{M,ng}}$, $I_{K_{M,ne}}$, $I_{Merga}$, and $I_{Mergib}$ were recorded from NG108-15, mouse sympathetic, and CHO cells, respectively. Deactivation was recorded in response to a voltage step to −50 mV from a holding potential of −20 mV ($I_{K_{M,ng}}$ and $I_{K_{M,ne}}$) or 0 mV ($I_{Merga}$ and $I_{Mergib}$). Means ± SEM of $\tau$ and contributions were obtained from deactivation relaxations recorded at −50 mV. Number of cells in parentheses; see Results.

$\tau$ contribution

\begin{table}
\centering
\begin{tabular}{lccc}
\hline
Current                  & Fast contribution & Slow contribution \\
\hline
$I_{K_{M,ng}}$ Total     & 117 ± 5 msec      & 658 ± 29 msec     & 3434 ± 159 msec \\
                          & 33.1 ± 2.1% (86/101) | 25.7 ± 1.7% (82/101) | 41.2 ± 2.2% (86/101) \\
                          & 33 ± 126 msec     & 2356 ± 247 msec   \\
Fast (WAY-insensitive)   & 104 ± 7 msec (37) | 456 ± 29 msec     & 2718 ± 156 msec \\
Slow (WAY-sensitive)     &                   & 41.7 ± 3.5% (34/36) | 58.3 ± 3.5% (36/36) \\
$I_{K_{M,ne}}$           & 93 ± 5.6 msec (n = 15) | 183 ± 10.6 msec | 1012 ± 85 msec \\
$I_{Merga}$              & 64.6 ± 2.4% (22/23) | 35.4 ± 2.4% (23/23) \\
$I_{Mergib}$             & 37.1 ± 19.4 msec | 112 ± 42.5 msec \\
                          & 70.8 ± 15.8% (3/3) | 29.2 ± 15.8% (3/3) \\
\hline
\end{tabular}
\end{table}

$I_{K(M_{ng})}$ (NG108-15 mouse neuroblastoma x rat glioma)

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Pharmacological separation of two components of the M-like ($I_{K(M_{nga})}$) current in NG108-15 mouse neuroblastoma x rat glioma cells. M-like currents recorded in two cells (A, B) as deactivating tail currents produced by voltage steps from −20 mV (holding potential) to −50 mV. In each cell, the control current had two different components, fast and slow, which could be blocked by WAY 123,398 and linopirdine, respectively. Insets on the right show the difference (blocker-sensitive) currents. The current in A consisted predominantly of the slow component (blocked by WAY 123,398), whereas that in B was predominantly fast and blocked by linopirdine.}
\end{figure}
Figure 2. Kinetic analysis of the M-like current in a NG108-15 mouse neuroblastoma x rat glioma cell. Total \( I_{K(M)} \) was activated by holding at \(-20\) mV and then deactivated by a 6 sec step (top record) to \(-50\) mV before (A) and after (B) addition of the erg-channel blocker WAY 123,398 (10 \( \mu \)M). In control (A) the deactivation tail was fitted (smooth line, superimposed) by the sum of fast \((t = 76\) ms, 254 pA) and two slow \((t = 340\) ms, 237 pA and 2124 ms, 70 pA) exponential curves. WAY 123,398 abolished both slow components without affecting the fast component (B, \( t = 76\) ms). C. Slow component obtained by subtracting the record shown in B from that shown in A was fitted (smooth line, superimposed) by the sum of two kinetic components \((t = 315\) and 1972 ms). All records were obtained from the same cell. Dashed lines denote zero current levels.

Figure 3. cDNA from mouse SCG, rat SCG, and chemically differentiated NG108-15 cells, was amplified using primers to the erg family of potassium channel genes or primers recognizing KCNQ2 and KCNQ3 potassium channel genes. Amplified products were obtained from all cell types but not from a negative control containing no template, indicating that members of these families are expressed by these cells. Sequence analysis revealed that the amplified product obtained from NG108-15 cDNA with primers to the erg genes was predominantly, if not exclusively, merg1. Analysis of the amplified product obtained using the KCNQ primers in both rat SCG and NG108-15 has shown these cells express both KCNQ2 and KCNQ3. M-1 kb ladder DNA size standards.
with PBS, blocked for 10 min with BSA, and permeabilized with 0.1% Triton X-100 for 5 min. Incubation with the anti-mergl antibody and labeling were carried out as described for the NG108-15 cells.

**Perforated-patch whole-cell recording.** Cells were bath-perfused with the solution of the following composition (in mM): 144 NaCl, 2.5 KCl, 2 CaCl$_2$, 0.5 MgCl$_2$, 5 HEPES, and 10 glucose, pH 7.4, with Tris base. Pipettes were filled with the "internal" solution containing 90 mM K acetate, 20 mM KCl, 40 HEPES, 3 MgCl$_2$, 3 mM EGTA, and 1 mM CaCl$_2$. The pH was adjusted to 7.4 with NaOH. Amphoterocin B was used to perforate the patch (Rae et al., 1991). The series resistance was not compensated because the error introduced was reasonably small. Thus, with the electrodes used (2-3 MΩ), the series resistance was 6–8 MΩ, and most of the currents were <0.5 nA, so the voltage error would be <5 mV. As confirmation that the voltage error was small, no correlation was found between deactivation time constants and initial current amplitude.

**Data acquisition and analysis.** Data were acquired and analyzed using pClamp software (version 6.0.3). Currents were recorded using an Axopatch 200A (or 200B) patch-clamp amplifier, filtered at 1 kHz, and digitized at 1–4 kHz. In current-clamp experiments, currents were injected, and membrane potential was recorded using an Axoclamp-2 amplifier. Activation curves were fitted by the Boltzmann equation: 

$$I(V) = I(\text{max}) \left(1 + \exp\left(V - V_{1/2}\right)/K_T\right),$$

where $I$ is current at the test potential, $I(\text{max})$ is the maximum current, $V_{1/2}$ is the membrane potential, and $K_T$ is the activation constant. Inhibition curves were measured from the change in the amplitude of the deactivation tail recorded at −50 mV. Each tail was fitted by one or more exponentials, and the tail amplitude was taken as the sum of the amplitudes of all components contributing to it after backfitting them to the beginning of the hyperpolarizing pulse. In cells that had the erg-type component, backfitting was necessary to exclude not only the (relatively fast) capacity transient, but also the brief rising phase of the tail caused by the deinactivation of erg-type channels. To include the fast component (when present) in the amplitude.

**RESULTS**

**Fast and slow M-like ($I_{\text{K(M,ng)}}$) currents in NG108-15 mouse neuroblastoma x rat glioma cells**

We recorded M-like currents ($I_{\text{K(M,ng)}}$) from 101 chemically differentiated NG108-15 cells using perforated-patch electrodes. Cells were carefully selected for their "neuron-like" appearance, i.e., large size and well-developed neuropil (Robbins et al., 1992). When studied with the conventional M-current voltage protocol, that is, by stepped hyperpolarization after prepolarization to approximately −20 mV (see Materials and Methods), currents showed characteristic M-like deactivation tails. However, the time course of these tail currents varied considerably from one cell to another. Figure 1 illustrates two extreme examples of this variation. Thus, in Figure 1A, deactivation during a 6 sec hyperpolarizing step was very slow, with an apparent "time-constant" of ~2 sec, whereas in Figure 1B, deactivation was complete within 2 sec.

Because a number of tumor cells (including neuroblastoma cells) have been reported to express HERG-like currents with...
Figure 5. Characteristics of the mergla current \(I_{\text{mergla}}\) expressed in CHO cells. \(I_{\text{mergla}}\) was activated by long (8 sec) depolarizing voltage steps from the holding level of \(-80\) mV (Aa) and deactivated by hyperpolarizing steps after its full activation by a prepulse to \(+50\) mV (Ba). Leak-subtracted steady-state \(I-V\) relationships obtained at the end of the depolarizing and hyperpolarizing pulses, respectively, are shown in Ab and Bb (open circles), and an "instantaneous" \(I-V\) relationship obtained at the beginning of the hyperpolarizing pulses for current deactivation is shown in Bb (filled circles). Activation (Ac) and deactivation (Be) time constants were plotted semilogarithmically, and \(I-V\) relationships were fitted by straight lines with \(I\) at 0 mV and the slope equal to \(1218 \pm 1\) msec and \(-0.013 \pm 0.001\) mV \(^{-1}\) in Ac and \(403 \pm 1\) msec and \(0.012 \pm 0.006\) mV \(^{-1}\) (open circles) in Be. C, Activation curve fitted by the Boltzmann equation at \(V_{1/2} = -5.9 \pm 0.6\) mV and \(k = 12.2 \pm 0.5\) mV. Records in Aa and Ba were from the same cell. In Ab, Ac, Bb, Be, and C the mean data are shown (vertical lines indicate SEMs) obtained from six and nine cells, respectively.

Because 10 \(\mu M\) WAY 123,398 produces a complete block of erg channels without affecting other K\(^+\) channels such as sympathetic neuron M channels (see below), we analyzed the fast and slow components of the deactivation tails in more detail by recording currents in the absence and presence of WAY 123,398. Figure 2 exemplifies the results obtained in 86 of 101 cells so examined. Here, the control current recorded in the absence of WAY 123,398 (Fig. 2A) showed three components, a fast component with a time constant of 76 msec, and a slower, biexponential component with time constants of 340 msec and 2.1 sec. WAY 123,398 (Fig. 2B) eliminated the slower component, leaving only the fast component (\(\tau\), 75 msec), whereas the difference (WAY-sensitive) current (Fig. 2C) showed only the biexponential slow component (\(\tau\), 315 msec and 2.0 sec). Thus, the time constants of the residual current recorded after application of WAY 123,398 and of the subtracted (WAY-sensitive) current accurately repro-

relatively slow rates of deactivation (Bianchi et al., 1998), we wondered whether these might contribute to the long deactivation tails. We tested this pharmacologically, using the HERG channel-blocking drug WAY 123,398 (Spinelli et al., 1993; Faravelli et al., 1996). As shown in Figure 1A, 10 \(\mu M\) WAY 123,398 blocked most of the long deactivation, leaving a residual fast component which was then eliminated by the M channel-blocking drug linopirdine (Aiken et al., 1995; Lamas et al., 1997). In contrast, the fast-deactivating current in Figure 1B was strongly blocked by linopirdine, leaving a slower component that was blocked in turn by WAY 123,398. Thus, comparison of the linopirdine- and WAY-sensitive currents (Fig. 1, inserts) showed that in fact each cell had two components to the deactivation currents, fast and slow, and that the overall time course of current deactivation was determined by their proportion. Furthermore, both components contributed to the sustained current recorded at \(-20\) mV.

Because 10 \(\mu M\) WAY 123,398 produces a complete block of erg channels without affecting other K\(^+\) channels such as sympathetic neuron M channels (see below), we analyzed the fast and slow components of the deactivation tails in more detail by recording currents in the absence and presence of WAY 123,398. Figure 2 exemplifies the results obtained in 86 of 101 cells so examined. Here, the control current recorded in the absence of WAY 123,398 (Fig. 2A) showed three components, a fast component with a time constant of 76 msec, and a slower, biexponential component with time constants of 340 msec and 2.1 sec. WAY 123,398 (Fig. 2B) eliminated the slower component, leaving only the fast component (\(\tau\), 75 msec), whereas the difference (WAY-sensitive) current (Fig. 2C) showed only the biexponential slow component (\(\tau\), 315 msec and 2.0 sec). Thus, the time constants of the residual current recorded after application of WAY 123,398 and of the subtracted (WAY-sensitive) current accurately repro-
duced the fast and slow components of the composite initial current. In this cell, the fast and slow components contributed 70 and 30%, respectively of the total tail current, and both contributed to the steady outward current at the holding potential as judged from the effect of WAY 123,398 on the holding current. On average, in the 86 cells expressing both currents, the fast- and slow-deactivating components contributed −33 and 67%, respectively, to the total tail current (Table 1). In the other 15 cells, the tail current showed only the slowly deactivating component and was fully suppressed by WAY 123,398.

mRNAs for merg1 and KCNQ2 and KCNQ3 in NG108–15 cells
The clearly distinguishable effects of linopirdine and WAY 123,398 on the tail currents illustrated in Figures 1 and 2 suggest that these might be composite currents, resulting from the deactivation of two different species of K⁺ channel: one composed of linopirdine-sensitive KCNQ2/3 subunits, or homologs thereof (Wang et al., 1998), and the other comprising a member (or members) of the erg family. We therefore sought evidence for the presence of transcripts of these channels by RT-PCR (see Materials and Methods). Transcripts for both erg and KCNQ2/3 were detected (Fig. 3).

Sequence analysis of 10 independent clones from the erg PCR showed that each clone contained erg1 DNA sequence (data not shown), suggesting that these cells express predominantly, if not exclusively, merg1 transcript (London et al., 1997). The KCNQ2/3 transcript contained mRNA for both KCNQ2 and KCNQ3. It may be noted in Fig. 3 that these transcripts were also present in...
Merg1 protein expression in chemically differentiated NG108–15 cells

The presence of mRNA transcripts does not necessarily correlate with the absence of any effect of WAY 123,398 on membrane currents recorded from these neurons (see below). Also, no immunostaining of differentiated NG108–15 cells was detected after exposure to an antibody raised against an N-terminal sequence unique to the short form of merg1 (merg1b), the expression of which is restricted to cardiac cells (London et al., 1997). This suggests that the protein tagged by the C-terminal antibody is the product of the long-form transcript merg1a (London et al., 1997; see also below).

Strong immunoreactivity for merg1-CT antibody was also detected in CHO cells transfected with merg1a cDNA. No staining was observed in untransfected cells, in cells transfected only with the GFP plasmid, or in cells treated with preabsorbed antibody (data not shown).

Slow \( \beta_{\text{K(M,ng)}} \) is mimicked by merg1a current \( \beta_{\text{merg1a}} \) expressed in CHO cells

The above results suggested that the slow component of the M-like current in NG108–15 cells might well be carried by merg1a channels. We tested this further by expressing merg1a cDNA in mammalian CHO cells. Figure 5 illustrates the resultant membrane currents. \( \beta_{\text{merg1a}} \) was activated by membrane depolarization to equal or positive to \(-40\) mV, but showed substantial inactivation during the (long) depolarizing command at potentials positive to \(0\) mV (Fig. 5Aa). As a result, the “steady-state” current–voltage curve was “bell-shaped” (Fig. 5Ab). The time course of this composite activation (accompanied by inactivation) could be described by two exponentials, accelerating strongly with depolarization (Fig. 5Ac). When the cell was hyperpolarized after a depolarizing prepulse to \(+50\) mV, there was a large transient enhancement of the current, caused by removal of channel inactivation, followed by a slower deactivation (Fig. 5Ba,b). Two deactivation components were detected that were strongly shortened by membrane hyperpolarization (Fig. 5Bc).

The mean activation curve deduced from tail currents followed a Boltzmann equation with \(V_{1/2} = -5.9 \pm 0.6\) mV and \(k = 12.2 \pm 0.5\) mV (Fig. 5C). However, when individual curves were fitted, they showed a great variation in \(V_{1/2}\) (range, between \(-27\) and \(13\) mV; \(n = 9\)) and small variation in \(k\) (range, between \(6.9\) and \(10\)). These results accord well with previous observations on merg1a currents in oocytes (London et al., 1997).

We next compared the properties of \( \beta_{\text{merg1a}} \) deactivation more closely with those of the WAY-sensitive slow component of \( \beta_{\text{K(M,ng)}} \), using the “standard” M current protocol, that is, currents were preactivated by holding at the depolarized potential of \(0\) mV (\(-20\) mV in the case of \( \beta_{\text{K(M,ng)}} \)), to avoid contamination by other, primarily Ca\(^{2+}\)-dependent, K\(^+\) currents and then deactivated by 6 sec step commands to various negative potentials. As shown in Figure 6, there was a close correspondence between the two. There were three main differences. First, deactivation of \( \beta_{\text{merg1a}} \) was preceded by a larger transient reactivation: this presumably reflected the greater steady-state inactivation of \( \beta_{\text{merg1a}} \) at \(0\) mV than that of slow \( \beta_{\text{K(M,ng)}} \) at \(-20\) mV. Second, the threshold for activation of \( \beta_{\text{merg1a}} \) was \(-10\) mV more positive than that for slow \( \beta_{\text{K(M,ng)}} \) (Fig. 6Ab,Bb): the reason for this is not known but may simply relate to different cell types. Third, whereas both showed a biexponential deactivation, the time constants for the two components of \( \beta_{\text{merg1a}} \) deactivation measured at \(-50\) mV were \(-40\%\) of those for deactivation of the slow \( \beta_{\text{K(M,ng)}} \) measured at the same potential (Table 1). The time constants showed a comparable voltage dependence (Fig. 6, compare Ac, Bc), so this

\[ \text{Figure 7. The slow component of the NG108–15 M-like current and the merg1a current are equally sensitive to WAY 123,398. Records in A and B show the slow component of } I_{\text{K(M,ng)}} \text{ and } I_{\text{merg1a}} \text{ deactivation tail currents recorded on stepping from } 0 \text{ to } -50 \text{ mV in the presence of increasing concentrations of WAY 123,398 (0, 0.3, 1, and 10 } \mu\text{M). Plots in C show mean percent inhibition of the tail currents (open circles, } I_{\text{K(M,ng)}}; \text{ n = 4}; \text{ filled circles, } I_{\text{merg1a}}; \text{ n = 6}). \text{ See Table 2 for fitted parameters.} \]
Table 2. Effects of K+ channel blockers on fast and slow $I_{K(M,ng)}$, $I_{mergla}$, and $I_{K(M)}$

<table>
<thead>
<tr>
<th>Blocker</th>
<th>Slow $I_{K(M,ng)}$</th>
<th>$I_{mergla}$</th>
<th>Fast $I_{K(M,ng)}$</th>
<th>$I_{K(M)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAY 123,398</td>
<td>0.4 ± 0.07</td>
<td>0.3 ± 0.02</td>
<td>no effect (10 μM)</td>
<td>no effect (10 μM)</td>
</tr>
<tr>
<td>nH</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.07</td>
<td>(37)</td>
<td>(9)</td>
</tr>
</tbody>
</table>

Azimilide

<table>
<thead>
<tr>
<th>IC50 (μM)</th>
<th>nH</th>
<th>IC50 (μM)</th>
<th>nH</th>
<th>IC50 (μM)</th>
<th>nH</th>
<th>IC50 (μM)</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5 ± 0.7</td>
<td>(4)</td>
<td>6.4 ± 0.3</td>
<td>(4)</td>
<td>31.1 ± 1.8</td>
<td>(4)</td>
<td>12.9 ± 0.7</td>
<td>(4)</td>
</tr>
<tr>
<td>0.9 ± 0.08</td>
<td>(5)</td>
<td>0.8 ± 0.03</td>
<td>(5)</td>
<td>0.9 ± 0.05</td>
<td>(5)</td>
<td>1.3 ± 0.09</td>
<td>(5)</td>
</tr>
</tbody>
</table>

THA

<table>
<thead>
<tr>
<th>IC50 (μM)</th>
<th>nH</th>
<th>IC50 (μM)</th>
<th>nH</th>
<th>IC50 (μM)</th>
<th>nH</th>
<th>IC50 (μM)</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.5 ± 1.5</td>
<td>(3)</td>
<td>35.7 ± 9.7</td>
<td>(3)</td>
<td>1542 ± 200</td>
<td>(3)</td>
<td>1279 ± 75</td>
<td>(3)</td>
</tr>
<tr>
<td>0.6 ± 0.05</td>
<td>(4)</td>
<td>0.6 ± 0.1</td>
<td>(4)</td>
<td>0.7 ± 0.07</td>
<td>(4)</td>
<td>0.8 ± 0.03</td>
<td>(4)</td>
</tr>
</tbody>
</table>

Linopirdine

<table>
<thead>
<tr>
<th>IC50 (μM)</th>
<th>nH</th>
<th>IC50 (μM)</th>
<th>nH</th>
<th>IC50 (μM)</th>
<th>nH</th>
<th>IC50 (μM)</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;&gt;30</td>
<td>(6)</td>
<td>&gt;30</td>
<td>(6)</td>
<td>1.2 ± 0.02</td>
<td>(6)</td>
<td>3.5 ± 0.2</td>
<td>(6)</td>
</tr>
<tr>
<td>1.2 ± 0.03</td>
<td>(4)</td>
<td>0.8 ± 0.04</td>
<td>(4)</td>
<td></td>
<td>(4)</td>
<td></td>
<td>(4)</td>
</tr>
</tbody>
</table>

TEA

<table>
<thead>
<tr>
<th>IC50 (mM)</th>
<th>nH</th>
<th>IC50 (mM)</th>
<th>nH</th>
<th>IC50 (mM)</th>
<th>nH</th>
<th>IC50 (mM)</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.7 ± 3.4</td>
<td>(6)</td>
<td>24.1 ± 3.6</td>
<td>(6)</td>
<td>1.1 ± 0.09</td>
<td>(6)</td>
<td>10.9 ± 0.7</td>
<td>(6)</td>
</tr>
<tr>
<td>0.8 ± 0.1</td>
<td>(6)</td>
<td>0.6 ± 0.1</td>
<td>(6)</td>
<td>0.6 ± 0.03</td>
<td>(6)</td>
<td>0.6 ± 0.02</td>
<td>(6)</td>
</tr>
</tbody>
</table>

Ba2+

<table>
<thead>
<tr>
<th>IC50 (μM)</th>
<th>nH</th>
<th>IC50 (μM)</th>
<th>nH</th>
<th>IC50 (μM)</th>
<th>nH</th>
<th>IC50 (μM)</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td>134 ± 6</td>
<td>(3)</td>
<td>492 ± 57</td>
<td>(3)</td>
<td>248 ± 26</td>
<td>(3)</td>
<td>393 ± 36</td>
<td>(3)</td>
</tr>
<tr>
<td>1.0 ± 0.04</td>
<td>(3)</td>
<td>1.0 ± 0.1</td>
<td>(3)</td>
<td>0.9 ± 0.07</td>
<td>(3)</td>
<td>0.8 ± 0.05</td>
<td>(3)</td>
</tr>
</tbody>
</table>

Mean ± SEM. Number of cells in parentheses. Inhibition constants (IC50) were determined from dose–response curves of the type illustrated in Figures 7 and 9. Effects on fast and slow currents in NG108-15 cells were determined from kinetic analysis of deactivation tails as illustrated in Figure 2, supplemented (where appropriate) by tests after inhibiting the slow component with WAY 123,398, as in Figure 9.

difference may be explained by the different activation thresholds and/or the different size of the voltage step (in four CHO cells using a prepulse protocol, the first and the second slow deactivation $r$ values for $I_{mergla}$ were slower after a prepulse to −20 mV compared with a prepulse to 0 mV, by 41 and 57%, respectively).

For comparison, we also examined the properties of currents generated by the short "cardiac" isoform merg1b expressed in CHO cells ($I_{merg1b}$; n = 3; data not shown). Whereas the voltage dependence of $I_{mergla}$ activation and deactivation were very similar to that of the slow $I_{K(M,ng)}$ and of $I_{mergla}$, both activation and deactivation of $I_{merg1b}$ were several times faster (Table 1), as previously reported in oocytes by London et al. (1997) (see also Lees-Miller et al., 1997). Hence, and in accordance with the lack of antibody staining mentioned above and the absence of merg1b mRNA expression in the nervous system (London et al., 1997), it is unlikely that merg1b channels contribute to slow $I_{K(M,ng)}$.

**Slow $I_{K(M,ng)}$ and $I_{mergla}$ show similar pharmacology**

We next compared the sensitivity of the slowly deactivating component of the NG108-15 current $I_{K(M,ng)}$ with that of CHO-expressed merg1a currents to some blocking drugs. As shown in Figure 7, both tail currents were blocked by the anti-arrhythmic drug WAY 123,398 with equal facility (IC50 values, 0.4 and 0.3 μM, respectively; Table 2). They were also equally sensitive to another anti-arrhythmic drug, azimilide (IC50 values, 6.4 and 6.5 μM, respectively; Table 2; Busch et al., 1998). $I_{mergla}$ was also inhibited by 9-amino-6,7-dihydropyridine (THA; IC50 value, 36 μM; Table 2), a compound that had previously proved unexpectedly potent in inhibiting the M-like current in NG108-15 cells (Robbins et al., 1992). In contrast, neither current was inhibited by the ganglionic M channel- and KCNQ2/3 channel-blocking agent linopirdine at concentrations up to 30 μM (Noda et al., 1998) reported an IC50 of 36 μM against the NG108-15 current, but this was measured from the depression of the composite current, and the Hill slope was rather shallow, so probably reflected its primary action on the fast current component, see below. Also, both currents were very insensitive to tetraethylammonium (TEA), with IC50 values of 17 and 24 mM (Table 2). Thus, $I_{mergla}$ provides a good match for the slowly deactivating component of $I_{K(M,ng)}$ both kinetically and pharmacologically.

**Fast $I_{K(M,ng)}$ is mimicked by the M current ($I_{K(M)}$) in mouse sympathetic neurons**

As noted above (Fig. 1), the fast component of $I_{K(M,ng)}$ was readily suppressed by 10 μM linopirdine. Since linopirdine blocks M currents in sympathetic ganglia (Lamas et al., 1997; Wang et al., 1998), this suggested that fast $I_{K(M,ng)}$ might correspond to the "true" (ganglionic-type) M current. We assessed this by comparing fast $I_{K(M,ng)}$ against the NG108-15 current, but this was measured from the depression of the composite current, and the Hill slope was rather shallow, so probably reflected its primary action on the fast current component, see below. Also, both currents were very insensitive to tetraethylammonium (TEA), with IC50 values of 17 and 24 mM (Table 2). Thus, $I_{mergla}$ provides a good match for the slowly deactivating component of $I_{K(M,ng)}$ both kinetically and pharmacologically.
Figure 8. Characteristics of the fast M-like $I_{K(M,ng)}$ current in a NG108–15 mouse neuroblastoma x rat glioma cell (A) and the M current ($I_{K(M)}$) in a mouse SCG neuron (B). The fast-deactivating component of $I_{K(M,ng)}$ (Aa) was recorded in the presence of 10 μM WAY 123,398 at different membrane potentials (holding level, −20 mV; step duration, 1 sec; interval, 10 sec; increment, −10 mV). Box, inhibition of the slow, WAY-sensitive component in this cell: currents before and after application of 10 μM WAY 123,398, left, and the difference current, right. For comparison (Ba), $I_{K(M)}$ is shown, obtained with the same voltage protocol. Deactivation tails were fitted by single exponential curves (smooth lines, superimposed). Leak-subtracted steady-state (filled circles) and instantaneous (open circles) I–V relationships for $I_{K(M,ng)}$ (Ab) and $I_{K(M)}$ (Ba) were obtained by measuring the current at the beginning and end of the voltage pulse. When the time constants for deactivation of $I_{K(M,ng)}$ (Ac) and $I_{K(M)}$ (Bc) were plotted semilogarithmically against membrane potential, S–V relationships were fitted by straight lines with $\tau$ at 0 mV and the slope equal to 271 ± 1 msec and 0.009 ± 0.0004 mV$^{-1}$ in Ac and 399 ± 1 msec and 0.013 ± 0.0009 mV$^{-1}$ in Bc. In Ab, Ac, Bb, and Bc the mean data are shown (vertical lines indicate SEM) obtained from 30 and 15 cells, respectively.

Pharmacological comparison of fast $I_{K(M,ng)}$ and mouse $I_{K(M)}$

Like fast $I_{K(M,ng)}$, mouse $I_{K(M)}$ was unaffected by 10 μM WAY 123,398. $I_{K(M)}$ recorded from 5 rat SCG neurons was also insensitive to this compound. Furthermore, both fast $I_{K(M,ng)}$ and mouse $I_{K(M)}$ were 1.5–2 orders of magnitude less sensitive to THA than were slow $I_{K(M,ng)}$ or $I_{erg}$ (IC$_{50}$ values, 1.5 and 1.3 mM; Table 2). This accords with the relative insensitivity of rat SCG $I_{K(M)}$ to THA reported previously (Marsh et al., 1990).

Figure 9 shows the responses of fast $I_{K(M,ng)}$ and mouse $I_{K(M)}$ to linopirdine and TEA. Whereas both currents were considerably more sensitive than slow $I_{K(M,ng)}$ or $I_{erg}$ to linopirdine, the neuroblastoma–glioma current was clearly more sensitive than the mouse SCG current (IC$_{50}$ values, 1.2 and 3.5 μM, respectively; Table 2). Likewise, fast $I_{K(M,ng)}$ was more readily blocked than the mouse $I_{K(M)}$ by TEA (see Discussion). Nevertheless, although not completely identical pharmacologically, fast $I_{K(M,ng)}$ and
mouse SCG $I_{K(M)}$ are clearly similar and together show an obvious difference from slow $I_{K(M,ng)}$ and $I_{mergla}$.  

**Both fast and slow $I_{K(M,ng)}$ and $I_{mergla}$, are inhibited through activation of M1 muscarinic receptors**

Activation of M1 muscarinic receptors inhibits both the total (composite) M-like current ($I_{K(M,ng)}$) in NG108–15 cells (Fukuda et al., 1988; Robbins et al., 1991, 1993), and the M current $I_{K(M)}$ in rat (Marrion et al., 1989; Bernheim et al., 1992) and mouse (Hamilton et al., 1997) sympathetic neurons. We therefore examined the effects of a muscarinic stimulant, oxotremorine-M (Oxo-M; 10 μM), on each component of $I_{K(M,ng)}$ as well as $I_{mergla}$ in M1 muscarinic receptor-transformed NG108–15 and CHO cells. Figure 10 shows that Oxo-M inhibited the slow $I_{K(M,ng)}$ (A) and $I_{mergla}$ (B). Such inhibitions were observed in seven of eight NG108–15 cells (mean inhibition, 42.3 ± 13.8%) and six of six mergla-expressing CHO cells (mean inhibition, 50.7 ± 10.8%). Inhibition of both slow $I_{K(M)}$ and $I_{mergla}$ was accompanied by a significant acceleration of their deactivation kinetics (Fig. 11): on average, the two components in slow $I_{K(M,ng)}$ and $I_{mergla}$ were shortened by 25.4 ± 10.0% and 34.6 ± 9.0% (n = 6) and 36.5 ± 4.8% and 27.8 ± 10.1% (n = 4), respectively.

Figure 12 shows examples of inhibitions of fast $I_{K(M,ng)}$ and mouse $I_{K(M)}$ by Oxo-M. Such inhibitions were observed in four of four NG108–15 cells (mean inhibition, 72.6 ± 13.8%; n = 4). As expected (Hamilton et al., 1997), similar inhibition was consistently observed in sympathetic neurons.

**Fast and slow $I_{K(M,ng)}$ control firing in NG108–15 cells**

The function of the M current is to act as a brake on repetitive firing. Thus, inhibition of the M current in sympathetic neurons, either by muscarinic agonists (Brown and Selyanko, 1985) or by an M channel-blocking agent (Wang et al., 1998), is associated with increased repetitive firing during depolarizing current injections. A similar effect has also been reported in M1-transformed NG108–15 cells after application of a muscarinic agonist (Robbins et al., 1993). However, in the latter case, it is not clear whether this results from inhibition of the fast or slow $I_{K(M,ng)}$ or both. We tested this by injecting long (7 sec) depolarizing currents into NG108–15 cells and then observing the effects of

---

**Figure 9.** Differential sensitivities of fast $I_{K(M,ng)}$ and $I_{K(M)}$ to linopirdine and TEA. The fast (WAY-insensitive) component of $I_{K(M,ng)}$ and $I_{K(M)}$ was recorded in response to 1 sec steps from the holding level of −20 to −50 mV, in the absence and presence of different concentrations of linopirdine (Aa, Ab) and TEA (Ba, Bb). C, Concentration dependences of inhibition of the two currents by linopirdine (a) and TEA (b). Smooth lines are the fits by the Hill equation. For the parameters of the fit see Table 2.
Figure 10. Muscarinic inhibition of slow $I_{K(M,n)}$ in a NG108-15 mouse neuroblastoma x rat glioma cell (A) and $I_{\text{merglia}}$ expressed in a CHO cell (B). Currents (Aa, Ba) were produced by holding at −20 mV (A) or 0 mV (B) and giving repeated steps (at 0.02 Hz in A and 0.025 Hz in B) to −50 mV for 6 sec. Both steady-state currents at the holding potentials and deactivation currents at the test potential were reduced by bath-application of oxotremorine-M (Oxo-M; 10 µM). In an NG108-15 cell, inhibition of $I_{K(M,n)}$ was preceded by a transient activation of a Ca$^{2+}$-activated K$^+$ current (Aa). Families of currents in b and c were obtained in response to incremental (−10 mV) hyperpolarizing voltage steps before (h) and during (c) action of Oxo-M. The insert in B shows superimposed current produced by voltage steps from 0 to −50 mV before and during the action of Oxo-M.

DISCUSSION

The main point emerging from this work is that the M-like current in NG108-15 cells ($I_{K(M,n)}$) is a composite current generated by at least two channel types: a fast-deactivating set of channels similar (but not quite identical) to those carrying the M current in mouse sympathetic neurons and tentatively identified as $KCNQ2/KCNQ3$ (Wang et al., 1998), and a slower-deactivating current probably formed from mergla (London et al., 1997). $KCNQ2$ and $KCNQ3$ are analogs of $KCNQ1$, which coassembles with $KCNE$ (minK) subunits to give the cardiac current $I_{Ks}$, the (slow component of the cardiac "delayed rectifier"), and mutation of which causes one form of the cardiac long QT syndrome (Yang et al., 1997). $KCNQ2$ and $KCNQ3$ have so far been detected only in brain and ganglia, and are implicated in a form of juvenile epilepsy (Biervert et al., 1998; Charlier et al., 1998; Schroeder et al., 1998; Singh et al., 1998; Yang et al., 1998). Mergla is one isoform of the mouse homolog of erg, originally cloned from a rat brain hippocampal cDNA library (Warmke and Ganetzky, 1994); mRNA for erg is found mainly in heart and brain (London et al., 1997; Wymore et al., 1997). Mutations of the human homolog $HERG$ give rise to a cardiac long QT syndrome (Curran et al., 1995; Sanguinetti et al., 1996), whereas mutations in $Drosophila$ erg are responsible for the seizure phenotype associated with hyperactivity in the flight motor pathway (Titus et al., 1997; Wang et al., 1997). Thus, both these channel types have been implicated selectively inhibiting fast and slow $I_{K(M,n)}$ with linopirdine and WAY 123,398, respectively. The depolarizing currents produced a short burst of repetitive firing in 20 of 22 NG108-15 cells tested and single action potentials in the remaining two cells. Figure 13 shows that 30 µM linopirdine (which blocked the fast current completely and inhibited the slow $I_{K(M,n)}$ by only 33%) produced a strong reduction in spike adaptation, whereas 10 µM WAY 123,398 had a much weaker effect. Neither linopirdine nor WAY 123,398 had any effect on spike repolarization.
Figure 11. Muscarinic inhibition of slow $I_{K(M,ng)}$ in an NG108-15 mouse neuroblastoma x rat glioma cell (A) and $I_{merg1a}$ expressed in a CHO cell (B) is accompanied by acceleration of their deactivation kinetics. Superimposed are deactivation tails obtained by stepping to $-50$ mV from $-20$ mV (A) or $0$ mV (B) in control (Con) and in the presence of $10 \mu M$ oxotremorine-M (Oxo). Smooth lines are double-exponential fits with time constants (indicated by arrows) equal to 276 and 1271 msec (Con) and 176 and 708 msec (Oxo) in A and 92 and 398 msec (Con) and 71 and 290 msec (Oxo) in B.

Figure 12. Muscarinic inhibition of fast $I_{K(M,ng)}$ in an NG108-15 mouse neuroblastoma x rat glioma cell (A) and $I_{merg1a}$ in a mouse sympathetic neuron (B). Fast-deactivating $I_{K(M,ng)}$ currents (A) are shown during 1 sec of hyperpolarization from $-20$ to $-50$ mV, before and during the action of oxotremorine-M (Oxo-M, 10 $\mu M$). Both currents were obtained in the presence of $10 \mu M$ WAY 123,398. (The effect of WAY 123,398 on the total $I_{K(M,ng)}$ recorded in this cell with a longer, 6 sec pulse, is shown in the box.) Currents in a mouse sympathetic neuron were obtained before and after addition of WAY 123,398 and WAY 123,398 + Oxo-M.

Our conclusion that two different channels are involved is based on: (1) the presence of mRNA transcripts and protein; (2) biophysical properties (voltage threshold and deactivation parameters); (3) sensitivity to potassium channel blockers; and (4) modulation of the channel by an agonist. Thus, we demonstrate the presence of mRNA and protein for $merg1$, and mRNA for $KCNQ2$ and $KCNQ3$, in NG108-15 cells. We show that the kinetics of $merg1a$ heterologously expressed in mammalian cells correspond closely to those of the slow $I_{K(M,ng)}$. We also show that the two are equally sensitive to the $merg$-selective blocking agents WAY 123,398 and azimilide, and insensitive to the ganglionic M current and $KCNQ2/3$ channel-blocking agent linopirdine. On the other hand, we find that the fast $I_{K(M,ng)}$ kinetically matches the mouse SCG $I_{K(M)}$, that these two are pharmacologically similar (although not quite identical), and that they can be distinguished from both slow $I_{K(M,ng)}$ and $I_{merg1a}$ by their greater sensitivity to linopirdine and insensitivity to WAY 123,398. We also show that both fast and slow components of $I_{K(M,ng)}$ are inhibited by stimulating $M_1$ muscarinic acetylcholine receptors and that the nature of the inhibition of these two components matches that for inhibition of mouse $I_{K(M)}$ and $I_{merg1a}$ respectively. $I_{K(M)}$ in rat ganglion cells has been ascribed to current through channels composed of heteromultimeric assemblies of expressed $KCNQ2$ and $KCNQ3$ subunits (Wang et al., 1998). This may also be true for $I_{K(M,ng)}$ in mouse ganglion cells and for the fast component of $I_{K(M,ng)}$ in NG108-15 cells, because we have found that both cell types show transcripts for these subunits. However, as yet, we cannot exclude a contribution from other, homologous, $KCNQ$ subunits. Furthermore, fast $I_{K(M,ng)}$ was distinctly more sensitive to TEA and to linopirdine than was mouse ganglion $I_{K(M)}$, suggesting that the subunit composition of the presumed-$KCNQ$ channels in these two cell types might differ.

The presence of functional $merg1a$ channels in these cells is not, in itself, particularly surprising, because mRNA transcripts have been identified in a number of neuroblastoma-derived cells in the control of excitability in vivo. This is the first example of these two channels forming overlapping and functionally similar components of membrane current.
NG108-15

Cell A

7 s
+0.23 nA
0.07 nA

Cell B

7 s
+0.22 nA
-0.03 nA

Control

0 mV

5 spikes

Control

0 mV

5 spikes

+ 30 µM linopirdine

16 spikes

+ 10 µM WAY 123,398

6 spikes

+ 10 µM WAY 123,398

19 spikes

20 mV

+ 30 µM linopirdine

23 spikes

Figure 13. Effects of inhibiting fast and slow $I_{\text{erg}}$ on firing in NG108-15 cells. Records show action potential trains in two NG108-15 cells (A, B) produced by long (7 sec) depolarizing pulses (top records) from the holding potential of -90 mV in the absence of drugs (Ab, Bc), in the presence of 30 µM linopirdine (Ab), 10 µM WAY 123,398 (Bb), or in the presence of both linopirdine and WAY 123,398 (Ac, Bc).

The transduction mechanism for $M_1$-mediated inhibition of $\text{KCNQ2/3}$ and $\text{merg1}$ is still unknown. Inhibition of $I_{\text{merg1}}$ and slow $I_{\text{KC}}$ was accompanied by accelerated deactivation, which may indicate the involvement of protein kinase C (PKC); in $\text{HERG}$ currents expressed in Xenopus oocytes, similar acceleration produced by thyrotropin-releasing hormone receptor activa-
tion was mediated by PKC (Barros et al., 1998). This would accord with an earlier proposal regarding the mechanism of inhibition of the M-like current in NG108–15 cells by bradykinin (Higashida and Brown, 1986). Although M<sub>4</sub> receptor activation in NG108–15 cells produces a strong elevation in intracellular [Ca<sup>2+</sup>] (Robbins et al., 1993), it is unlikely that Ca<sup>2+</sup> could be a messenger for muscarinic inhibition of slow I<sub>K(M,avg)</sub> or I<sub>Mergl</sub> because, in CHO cells, the Ca<sup>2+</sup>-ionophore ionomycin (5 μM) produced an insignificant reduction in I<sub>Mergl</sub>, (to 89.4 ± 9.7% of control; n = 3). As a control for the effectiveness of ionomycin in these cells, it caused a complete block of Kv1.2 channels expressed in CHO cells when recorded in perforated-patch or cell-attached configurations; direct application of 500 nM Ca<sup>2+</sup> blocked Kv1.2 channels when recorded in the inside-out configuration (A. A. Selyanko and J. K. Hadley, unpublished observations).

Do products of erg genes contribute to M-like currents in other neurons? Transcripts for mergl (London et al., 1997), and for the rat homologs erg1 and erg3 (Shi et al., 1997; Wymore et al., 1997) are present in mammalian brain. Furthermore, both expressed merglA currents and the slow, presumed-merglA component of the M-like current in NG108–15 cells were inhibited by stimulating M<sub>4</sub> muscarinic receptors, so they could contribute to muscarinic-inhibitable M-like currents previously recorded in central neurons. True, the presence of mRNA transcripts may not be token in the assembly of functional channels: thus, no appropriate erg-like component of membrane current could be recorded from mouse or rat sympathetic neurons, in spite of the presence of mRNAs (Shi et al., 1997; see also this paper), nor could we detect mergl immunity. However, this may not be the case for other mammalian neurons. For example, the M-like current recorded from isolated rat cortical neurons has been reported to be an order of magnitude less sensitive to linopirdine than either the ganglionic or hippocampal cell current (Noda et al., 1998; cf. Aiken et al., 1995; Lamas et al., 1997; Schnee and Brown, 1998). Although other explanations are possible, our findings suggest that this might arise from a contribution by erg channels to the cortical neuron current. In view of the significance of M-like channels as potential targets for cognition-enhancing drugs (Zaczek and Saydoff, 1993), further information regarding the degree of heterogeneity in the molecular composition of the channels underlying M-like currents in different neurons would be helpful.

REFERENCES


London B, Trudeau MC, Newton KP, Beyer AK, Robertson GA (1997) Two isoforms of the mouse e<sub>h</sub>erg<sub>a</sub>-a-go-go-related gene coassemble to form channels with properties similar to the rapidly activating component of the cardiac delayed rectifier K<sup>+</sup> current. Circ Res 81:780–787.


Marsh SJ, Hubbard A, Brown DA (1980) Some actions of 9-amino-1,2,3,4-tetrahydroacridine (THA) on cholinergic transmission and


