Selective Electrical Silencing of Mammalian Neurons In Vitro by the Use of Invertebrate Ligand-Gated Chloride Channels

Eric M. Slimko, Sheri McKinney, David J. Anderson, Norman Davidson, and Henry A. Lester

1Computation and Neural Systems Program and 2Division of Biology, California Institute of Technology, Pasadena, California 91125

Selectively reducing the excitability of specific neurons will (1) allow for the creation of animal models of human neurological disorders and (2) provide insight into the global function of specific sets of neurons. We focus on a combined genetic and pharmacological approach to silence neurons electrically. We express invertebrate ivermectin (IVM)-sensitive chloride channels (Caenorhabditis elegans GluCl α and β) with a Sindbis virus and then activate these channels with IVM to produce inhibition via a Cl⁻ conductance. We constructed a three-cistron Sindbis virus that expresses the α and β subunits of a glutamate-gated chloride channel (GluCl) along with the green fluorescent protein (EGFP) marker. Expression of the C. elegans channel does not affect the normal spike activity or GABA/glutamate postsynaptic currents of cultured embryonic day 18 hippocampal neurons. At concentrations as low as 5 nM, IVM activates a Cl⁻ current large enough to silence infected neurons effectively. This conductance reverses in 8 hr. These low concentrations of IVM do not potentiate GABA responses. Comparable results are observed with plasmid transfection of yellow fluorescent protein-tagged (EYFP) GluCl α and cyan fluorescent protein-tagged (ECFP) GluCl β. The present study provides an in vitro model mimicking conditions that can be obtained in transgenic mice and in viral-mediated gene therapy. These experiments demonstrate the feasibility of using invertebrate ligand-activated Cl⁻ channels as an approach to modulate excitability.

Key words: silencing; excitability; hippocampal neurons; chloride channels; Sindbis virus; transfection of neurons; EYFP; ECFP

This paper focuses on an approach to understanding the role of individual neuronal cell types in development, information processing, and behavior. Several investigators have suggested that such information can be obtained by using gene transfer in vitro, or transgenic animals, to produce spatially and temporally controlled inactivation of specific neurons (White et al., 2001a). Recent studies report the use of transcriptional induction of various K⁺ channels (Johns et al., 1999; Falk et al., 2001), and this method silences Drosophila neurons or muscle in vivo (Paradis et al., 2001; White et al., 2001b; Nitabach et al., 2002), providing useful insights into the role of defined neuronal populations. We previously studied the possibilities for neuronal silencing by using expressed G-protein-gated inwardly rectifying K⁺ (GIRK) channels (Ehrengruber et al., 1997), weakly inwardly rectifying K⁺ channels (Nadeau et al., 2000), and neuron-restrictive silencing factor (Nadeau and Lester, 2002). However, there are indications that K⁺ channels may cause unwanted side effects, such as apoptosis caused by sustained K⁺ efflux, in the cultured mammalian CNS neurons that were used to test this strategy (Nadeau et al., 2000).

The emerging genetic techniques seem to present some advantages over previous exclusively pharmacological strategies. Target areas and nuclei indeed have been inactivated in vivo via the focal application of a GABA agonist, muscimol (Jasnow and Huhman, 2001; Maren et al., 2001). This procedure has been valuable in determining the role of the amygdala and hippocampus in fear conditioning (Helmstetter and Bellgowan, 1994; Muller et al., 1997; Holt and Maren, 1999; Wilensky et al., 1999, 2000). However, the muscimol strategy suffers from limitations such as the inability to know exactly which neurons were silenced, the inability to silence specific cell types in a given brain region, the inability to silence distributed but functionally related neuronal populations, and the irreproducible localization of focal injections. Also, specific neuronal populations have been ablated irreversibly (Kobayashi et al., 1995; Sawada et al., 1998; Watanabe et al., 1998; Isles et al., 2001), but further insights could arise from a reversible technique.

We suggest a strategy that combines the genetic and acute pharmacological manipulations. Because GABA and glycine are the major inhibitory neurotransmitters in the brain and because GABAA and glycine receptors inhibit activity by activating a Cl⁻ conductance, we have explored the possibility of using ligand-activated Cl⁻ channels as silencing genes. The glutamate-gated chloride channel (GluCl) family of ligand-gated Cl⁻ channels, found in several invertebrate phyla, is the target for various pesticides, anthelmintic, and antiparasitic drugs, including ivermectin (IVM) (Cully et al., 1994). GluCl channels are part of the nicotinic receptor superfamily, characterized by four transmembrane segments and a large N terminus, and are most similar to GABAA and glycine receptors. Glutamate is the putative ligand for these channels. No GluCl channels have been detected yet in mammals (Martin et al., 1998; Xue, 1998).

The GluCl channels may represent a promising set of silencing genes. We have used a recombinant Sindbis virus to express these channels in cultured neurons and then tested whether the ex-
pressed channels can be activated with high sensitivity and high selectivity by IVM. Indeed, we do find that IVM at low nanomolar concentrations induces a large Cl− conductance in some infected neurons, effectively clamping the membrane at a potential near the original resting potential. As a result, neither current injection nor focal application of excitatory transmitter elicits action potentials. We also studied possible unwanted additional effects of either GluCl channel expression or IVM application and found these potential side effects to be minimal or nonexistent. The inducible and reversible nature of this inactivation, which we term the GluCl/IVM method, represents a potential powerful new technique for use in probing the contributions of specific regions of the nervous system to the global function.

MATERIALS AND METHODS

Viral vectors and preparation. Wild-type Caenorhabditis elegans GluClα and GluClβ genes (Cully et al., 1994) cloned into pBluescript II SK− were a kind gift from Merck Research Laboratories (Whitehead, NJ); the Sindbis vector pSinRep5dsGEGFP was a kind gift from Drs. H. Nawa and M. Kawamura of Niigata University (Niigata, Japan). The oligos pSG1 (5′-6CG ACG TCA TCT CTA CGG TGG GCC TCC TAA ATA GTA TGT TAA ACG CAT G-3′) and pSG2 (5′-6CG TGG TTA AAA CTT TTA AGG ACC ACC GTA GAG ATG ACG TCG CAT G-3′) were annealed and ligated into the SphI site of pSinRep5dsGEGFP, resulting in pSinRep5dsStgEGFP. GluClα was amplified by PCR with the primers 5′-ATA GAT CGT CTA TCA CTG CAT AAA T-3′ and 5′-TAG ATT CAC GAC TAA GCA TCC TCG ATG A-3′ and cloned into the MluI and AatII sites of pSinRep5StgEGFP, resulting in pSinRep5StgGluClαEGFP. GluClβ was amplified by PCR with the primers 5′-AAT GCA GCA TGC TAT AGC CTG TAG TAT GAT GAT TGG TTC A-3′ and cloned into the SphI site of pSinRep5StgGluClαEGFP, resulting in pSinRep5StgGluClβEGFP (Fig. 1). This plasmid was used to generate recombinant Sindbis virus according to the manufacturer’s instructions. Briefly, RNA was transcribed from this plasmid and used to help generate IVM-induced conductance in single voltage- and current-clamped neurons by using a Picospritzer with a U-tube system providing the wash (Khakh et al., 1995).

Statistics. Pooled data are shown as means ± SEM.

RESULTS

Initial experiments were performed on transfection of the GluClα and β subunits into human embryonic kidney (HEK) cells. Robust responses to IVM (500 nM) were detected only in cells that expressed both the α and β subunits (Fig. 2A,B). Unlike recordings from Xenopus oocytes (Cully et al., 1994), the current was transient; however, when we used successive voltage ramps to measure the conductance and reversal potential of the current, we found that the conductance remained relatively constant and the reversal potential changed after the application of IVM (Fig. 2C,D). In experiments that are not shown, we found that the IVM-induced conductance was stable for at least 30 min. These results encouraged us to design and construct the Sindbis virus, vSinGluClαβEGFP, which expressed three genes: GluClα, GluClβ, and green fluorescent protein (GFP) as a reporter for infected cells.

The GluCl/IVM method suppresses neuronal excitability

We infected 10- to 14-d-in-culture rat E18 hippocampal cultures with vSinGluClαβEGFP or the control virus vSinEGFP. As expected from previous studies (Khakh et al., 2001), the cultures were infected readily by either Sindbis virus, showing robust GFP expression in 20–50% of the neurons as early as 6 hr after infection. Previous studies also show that Sindbis-infected neurons die after ~48 hr, presumably because the virus recruits all of the ribosomes of the cell and thereby effectively shuts off host

**Figure 1.** Modified pSinRep5 construct, pSinRep5StgGluClαEGFP, designed to express three genes with three subgenomic promoters: the GluClα subunit, the GluClβ subunit, and the reporter EGFP. The resulting Sindbis virus is vSinGluClαβEGFP.

**Figure 2.** Both GluClα and β are required for functional channels in HEK 293 cells. A, Voltage-clamp record of a HEK 293 cell transfected with GluClα and EGFP shows that this subunit alone does not respond to applications of 500 nM IVM. Five of five cells showed no response to this subunit alone. B, Voltage-clamp record of a HEK 293 cell transfected with GluClα and β subunits shows a transient IVM-induced current. Five of five cells transfected in this manner showed a robust response to IVM. C, Voltage ramps from ~90 to ~40 mV before (arrow), during, and after the application of 500 nM IVM. The increasing slopes show the IVM-induced conductance. The waveforms also show a clear change in reversal potential during the development of the conductance. D, Input resistance of the cell in C. A conductance develops and then remains relatively constant after the application of IVM.

HEPES, 3 ATP, 3 phosphocreatine, and 0.3 GTP, pH 7.2, at 215 mOsm. Whole-cell voltage clamp was maintained by using an Axopatch-1D amplifier controlled by a personal computer running pClamp 8 software via a Digidata 1200 interface (Axon Instruments, Foster City, CA). Data were filtered at 2 kHz and digitized at 5 kHz. Drugs were applied focally to single voltage- and current-clamped neurons by using a Picospritzer with a U-tube system providing the wash (Khakh et al., 1995).

Electrophysiology. The bath solution contained (in mM): 110 NaCl, 5.4 KCl, 1.8 CaCl2, 0.8 MgCl2, 10 HEPES, and 10 d-glucose, pH 7.4, with an osmolality of 230 mOsm. Patch pipettes were filled with a solution containing (in mM): 100 K-gluconate, 0.1 CaCl2, 1.1 EGTA, 5 MgCl2, 10...
protein synthesis (Frolov and Schlesinger, 1994; Perri et al., 2000); therefore, we performed our experiments at 24 hr after infection. Neurons that were infected with vSinGluClαβEGFP and vSinEGFP had morphology indistinguishable from uninfected controls at this stage and appeared to be healthy (data not shown).

In ~20% of GFP-positive cells that were infected with vSinGluClαβEGFP, we detected a robust conductance increase with the application of 5–500 nM IVM (Fig. 3A–C, bottom panels). The time to activate this current was dose-dependent; the time constant decreased from 500 sec at 5 nM to 6 sec at 500 nM. Surprisingly, the maximal conductance did not seem to vary in this concentration range. Also shown in the top panels of Figure 3 is the robust silencing effect of this conductance increase. Spontaneously firing cells subject to the application of IVM stopped firing action potentials, and the membrane potential moved to values between −50 and −65 mV, which is near the value of EC50 for the solutions that we used. Importantly, vSinEGFP-infected neurons and uninfected neurons showed no responses to IVM at these concentrations (data not shown).

Glutamate is the major excitatory transmitter in the brain, and brief applications of glutamate strongly elicit action potentials in hippocampal neurons (Storm-Mathisen, 1981; Crunelli et al., 1983). The major goal of the GluCl/IVM method is to insure that, once a GluClαβ-positive cell is activated by IVM, it no longer fires action potentials in response to excitatory input. Figure 4A demonstrates this effect. The left panel shows that an infected neuron fires volleys of action potentials in response to 10 msec pulses of 100 μM glutamate; the right panel shows that, in the presence of 5 nM IVM, the same neuron no longer responds to identical glutamate pulses. Below (see Fig. 8), we show that neurons expressing this invertebrate glutamate receptor display no changes in glutamate responses.

Additionally, neurons that are infected with vSinGluClαβEGFP and activated with IVM show extreme reductions in impulse firing elicited by direct current injection. The top panel of Figure 4B shows current-clamp records from a vSinGluClαβEGFP-infected neuron. Increasing current pulses (up to 30 pA) elicit current-dependent firing. The bottom panel shows records from a similar neuron, incubated with 5 nM IVM. This neuron does not respond at all to the same current injection protocol. Moreover, injection of up to 200 pA (data not shown) failed to elicit a spike.

Figure 4C shows the same experiments for an uninfected control cell; 5 nM IVM did not affect the excitability. This lack of IVM effect was observed in all of 103 uninfected or 55 vSinEGFP-infected neurons, respectively.

**Sindbis expression produces highly variable results**

Previous reports show large cell-to-cell variability in expression levels, based on measurements that used dual promoters to express an ion channel subunit with the Sindbis virus (Okada et al., 2001). We expected similar cell-to-cell variability in our experiments. Unexpectedly, we found that the major variability in IVM responses occurred between culture dishes rather than within neurons in a given culture (Fig. 5). Each point in Figure 5 represents the average of five infected neurons in a single infected culture. Some cultures respond well, whereas others do not. We could observe no clear morphological differences or GFP fluorescence intensity differences between cultures that expressed well and those that expressed poorly. We attempted, with no success, to minimize this variability by varying the age of the cultures, the time after infection, temperature and position in the incubator, preparation of the virus, sonication of the virus, and glutamate concentration in the culture medium. In control dishes that were infected with vSinEGFP, there was no significant difference between the IVM+ and IVM− input conductance in any culture that was tested (data not shown). Because of this culture-to-culture variation, we selected only cultures that had neurons responding well to IVM for the measurement studies presented above. We found similar dish-to-dish variability in HEK 293 cultures that were infected with vSinGluClαβEGFP, but not in HEK cultures transfected with both plasmids encoding the GluCl
subunits; therefore, we believe that this variability is a characteristic of the Sindbis expression system rather than an inherent limitation of the GluCl/IVM method.

In Nupherin-mediated transfection, all fluorescent cells respond to IVM

We sought an expression method that could overcome the variability of the Sindbis system. We used the newly developed Nupherin-neuron transfection system to transfect GluCl channels into hippocampal cultures. In these experiments we modified the GluCl coding sequence to include fluorescent proteins (yellow fluorescent protein, YFP; cyan fluorescent protein, CFP) in the M3–M4 loop of each construct (GluClα and β subunits, respectively, as shown in Fig. 6A). Figure 6C–E shows images from an exemplar hippocampal culture. Each of 30 fluorescent cells in three cultures responded to 5 nM IVM with a conductance >30 nS, similar to the results with neurons in responsive Sindbis-infected cultures (Fig. 6B). This figure shows that neurons transfected with the GluClα subunit alone do not develop a conductance in the presence of IVM. The variability between cultures is substantially less with Nupherin-mediated expression than with Sindbis-mediated expression.

IVM-induced Cl⁻ conductance is reversible

In previous reports IVM has been described as acting irreversibly on GluCl channels expressed in Xenopus oocytes (Cully et al., 1994) on a time scale of several minutes. Ligand-binding experiments show that IVM does dissociate from its receptor (presumably the GluCl channels), with a rate constant of 0.005–0.006/min (Cully and Paress, 1991). Therefore, we sought to measure the reversibility of IVM action on a time scale of several hundred minutes. We infected cultures of hippocampal neurons, incubated in 5 nM IVM, and we measured the input conductance of the neurons to verify that the channels were activated. We then washed the cultures and recorded the input conductance after 1 or 8 hr. Figure 7A summarizes these results. At 1 hr the conductance has not changed significantly from the activated conductance, whereas by 8 hr the conductance has returned nearly to baseline. The final bar shows that IVM subsequently can reactivate the channels. Formally, it is not possible to distinguish between the possibility that the reversibility is caused by IVM washing off the GluCl receptors or the possibility that the reversibility is attributable to receptor turnover, but the important feature here is that the technique appears to be reversible in terms of electrical activity.
Figure 8. Expression of GluCl does not alter glutamate- or GABA-evoked currents. A. Evoked currents were measured in GluCl-infected and uninfected neurons with 10 msec agonist puffs (100 μM GABA, 1 mM glutamate). The amplitude, rise time, and decay time were measured. The plot shows these three parameters relative to the measurements in uninfected cells. GABA responses are shown on the left and glutamate responses on the right. For each of the two ligands the bars represent data pooled from 10 cells from two different cultures. B, C. Analysis of GABA mIPSCs in the absence (B) and presence (C) of 5 mM IVM. The peak amplitude histogram (top) and decay time histogram (bottom) are shown. The amplitude data are shown in 10 pA bins, and the decay time data are shown in 10 msec bins. Note that there is no apparent difference in histogram shape between the 0 and 5 mM IVM case for either measurement. In B, the data are pooled from 20 events each from five neurons in one culture; in C, the data are pooled from 30 events each from five neurons in one culture.

Neither GluCl expression nor IVM application affects other synaptic properties

In experiments to examine possible unwanted additional effects of IVM, we used 5 mM IVM, because this concentration produces adequate silencing in most neurons. Because of the homology between GluCl channels and GABA_α receptors, we were concerned that one of these subunits might heteromultimerize with native GABA_α receptor subunits. We therefore compared the amplitude, rise time, and decay time of GABA-evoked responses. The left panel of Figure 8A shows that these three parameters do not differ significantly between infected and uninfected cells, providing confidence that heteromultimerization with GABA subunits did not take place or at least had no functional consequences. After measuring the evoked responses, we applied 5 mM IVM to these cultures to ensure that these cultures were expressing the GluCl receptor at high levels.

The GluCl α and β heteromultimer is a glutamate receptor (Cully et al., 1994). We were concerned that this response might add to or distort the endogenous glutamate response of the neuron. The right panel of Figure 8A addresses these concerns in an experiment that used Sindbis-mediated expression: the glutamate response did not change significantly between uninfected and GluClαβ-expressing neurons. As above, we used only cultures that responded well to IVM for these experiments. These experiments were performed with C6Cl in the internal recording solution and were voltage clamped at −60 mV so that any additional conductance caused by glutamate activation of GluCl would result in an increase in amplitude or perhaps an increase in decay time. In infected cultures that were treated with CNQX and APV to block endogenous glutamate receptors, we found that steady application of 10 μM glutamate (the estimated steady concentration of glutamate in CSF) produced no detectable conductance increases (<0.1 nS). Steady application of 2 mM glutamate, however, produced slow, large (~30 nS) conductances.

IVM has various effects on other ion channels, including GABA (Krusk and Zemkova, 1994), P2X_2 (Khakh et al., 1999), and neuronal AChR (Krause et al., 1998). These effects occur with concentrations of IVM higher than 5 mM. However, we chose to study what we regard as the most likely side effect, GABA potentiation, with our desired IVM concentration of 5 mM. Figure 8B presents histograms of the amplitude and decay time of the GABAergic synaptic miniature IPSCs (mIPSCs) recorded from uninfected neurons in the presence of the glutamate receptor blockers CNQX and APV and the Na^+ channel blocker TTX. Figure 8C presents the same type of data, with the addition of 5 mM IVM in the recording medium. There is no apparent potentiation of GABA receptors at this concentration of IVM.

DISCUSSION

We describe a procedure that combines advantages of genetically based and pharmacologically based silencing by inhibitory ion channels. Our results demonstrate that the GluCl/IVM method modifies neuronal excitability in an inducible and reversible manner. Expression of GluCl alone seems to leave the excitability of the neuron unaltered, and IVM application to uninfected neurons within the effective range for silencing GluCl-expressing neurons never produces a detectable conductance. However, many GluCl-expressing neurons display a reduction in excitability when exposed to low concentrations of IVM. The reduction in excitability silences many neurons, and this silencing can be reversed by ~8 hr of washing. These are the desired results of the GluCl/IVM silencing method.

With the Sindbis virus construct there was an inherently high level of variability of expression among infected cells. Despite numerous attempts to understand and control this variability, we found no parameters to explain it. Both HEK cells transfected with the GluCl α and β subunits and *Xenopus* oocytes injected with RNA for this channel nearly always show expression, reinforcing our belief that this variability results from an aspect of Sindbis viral biology. Nupherin-mediated expression yields more consistent data, supporting this interpretation. Because our long-term goal involves transgenic methods to express the GluCl channels, the variability does not appear to be a source of significant concern for the GluCl/IVM silencing method.

Neurons expressing the channel at a high level are silenced strongly by 5 mM IVM. This low concentration is important, for previous reports show that IVM at considerably higher concentrations in the mammalian brain is toxic. Studies done with radiolabeled IVM in blood–brain barrier-impaired mice indicate that the toxic concentration of IVM in the brain is ~500 mM (Schinkel et al., 1994), which is two orders of magnitude above the concentration we are using. This gives us some confidence that in vivo applications of the GluCl/IVM method may succeed.

A related complexity is the delivery of IVM to the brain. The blood–brain barrier contains mechanisms, primarily involving *mdr1*, that appear to pump IVM out of the brain, and this might prevent systemically applied IVM from reaching GluCl channels in the brain. However, a clinically relevant intraperitoneal dose, 0.2 mg/kg, results in 1.5 ng/gm IVM in brain tissue (Schinkel et al., 1994), or ~2 mM. This is very close to the concentration we believe to be necessary for silencing neurons expressing GluCl. Perhaps in vivo applications of the GluCl/IVM method will...
require relatively simple methods of IVM delivery to the animal. Of course, more sophisticated options exist for easing drug delivery, such as using blood–brain disrupting drugs (Jette et al., 1995) or using mice with disrupted drug pumping at the blood–brain barrier, as the foundation for transgenic mice expressing this channel.

Previous reports suggest that IVM “irreversibly” activates GluCl channels, an unusual situation for ligand-gated channels. The impression of irreversibility may arise from the limited time scale of previous experiments on Xenopus oocytes or from drug retention by the large yolk volume of the cells. Data from our washout experiments (Fig. 7), suggesting reversibility after 8 hr, are approximately consistent with the disassociation constant determined in biochemical binding experiments (Cully and Parress, 1991). Both the onset and recovery from silencing will depend on the pharmacokinetics of IVM in the animal; for instance, IVM can be stored in the fat.

In addition to the channel-based silencing strategies noted in the introductory remarks, other investigators have used genetic manipulations of synaptic transmission, for instance by using tetanus toxin (Baines et al., 2001). It is unclear whether tetanus toxin-based procedures are reversible. Additionally, exogenous expression of K+ channels has been used to modulate biochemical signaling pathways in HEK 293 cells (Holmes et al., 1997).

There are several potential applications for the GluCl/IVM method, or for improved versions, in research and in therapeutics. One application would involve cell-specific expression of GluCl channels under the control of appropriate promoter(s). This strategy may enable genetic manipulation of excitability at selected times in development and in specific regions in the CNS. The requirement for both the GluCl α subunit and the GluCl β subunit at first may seem like a complication; however, this gives the experimenter the flexibility to use different promoters to drive the two genes so that only neurons in which both promoters are active will be silenced with the administration of IVM. Additionally, it may be possible to construct viruses with these two genes that can be used in vivo. Stereotoxic injection of such viruses may circumvent the need for promoters that drive expression in specific brain nuclei. From a clinical standpoint, a targeted and controlled “excitability modulator” may form the basis for new treatments of epilepsy, chronic pain, or other diseases arising from excess neuronal activity. It may be possible to titrate silencing by varying the dose of IVM.

REFERENCES


