FEBS 26471

FEBS Letters 528 (2002) 77–82

Selective elimination of glutamate activation and introduction of fluorescent proteins into a *Caenorhabditis elegans* chloride channel

Ping Li[a], Eric M. Slimko[b], Henry A. Lester[a,*]

[a] *Division of Biology, M/C 156-29, California Institute of Technology, 1200 E. California Boulevard, Pasadena, CA 91125, USA*
[b] *Computation and Neural Systems Program, California Institute of Technology, Pasadena, CA 91125, USA*

Received 1 July 2002; revised 7 August 2002; accepted 8 August 2002

First published online 28 August 2002

Edited by Maurice Montal

Abstract **Glutamate-gated chloride (GluCl) channels from invertebrates can be activated by ivermectin (IVM) to produce electrical silencing in mammalian neurons. To improve this GluCl/IVM strategy, we sought to mutate the *Caenorhabditis elegans* GluCl channels so that they become insensitive to glutamate but retain their sensitivity to IVM. Based on structure–function studies of nicotinic acetylcholine receptor superfamily members, we tested in oocytes 19 point mutants at 16 residues in the β-subunit likely to be involved in the response to glutamate. Y182F reduces the glutamate response by greater than six-fold, with little change to IVM responses, when coexpressed with wild-type (WT) GluCl α. For GluCl αβ(Y182F), the EC_{50} and Hill coefficient for glutamate are similar to those of WT, indicating that the mutant decreases the efficacy of glutamate, but not the potency. Also, fluorescent proteins (enhanced green fluorescent protein, enhanced yellow fluorescent protein, enhanced cyan fluorescent protein; XFP) were inserted into the M3–M4 loop of the GluCl α, β and β(Y182F). We found no significant functional difference between these XFP-tagged receptors and WT receptors. The modified GluCl channel, without glutamate sensitivity but with a fluorescent tag, may be more useful in GluCl silencing strategies.**

Key words: Ion channel; Electrical silencing

1. Introduction

Several recent studies describe expression of exogenous ion channels in order to change the electrical properties of neurons [1–8]. Our laboratory has developed a procedure that employs the glutamate-gated chloride (GluCl) channels from *Caenorhabditis elegans* [9–11]. Because the anthelminthic ivermectin (IVM) activates these channels at concentrations too low to affect other mammalian ion channels, it is possible to envision a procedure that selectively silences the target neurons. The GluCl channels would be expressed in the target neurons either by local injection of viral vectors or in transgenic animals under cell-specific promoters.

The GluCl/IVM technique could be improved in two ways. First, the GluCl channels should be rendered insensitive to glutamate, which is present in CSF and released during synaptic transmission. Second, the expression of the GluCl channels should be physically verifiable by a technique that has higher throughput than electrophysiology. Fluorescent proteins are widely employed for such purposes; and the channels could be fused to fluorescent proteins.

We chose the GluCl channels in part because they are members of the nicotinic receptor superfamily, which has been widely studied by mutagenesis [12]. Therefore several precedents suggest ways to re-engineer the GluCl channels [13–15]. For the goal of eliminating glutamate responses while retaining IVM responses, one considers the residues in the N-terminal region known to bind agonists (Fig. 1) [16,17]. For the goal of introducing fluorescent proteins, previous results suggest that the intracellular loop between the M3 and M4 domains is the appropriate region [18].

2. Materials and methods

2.1. Mutagenesis

Wild-type (WT) *C. elegans* GluCl α and GluCl β genes [11] cloned into pBluescript II SK^+ were a gift from Merck Research Laboratories. In our present study [9], they were cloned into pcDNA 3.1 (ClonTech, Palo Alto, CA, USA) with *Kpn*I and *Not*I. Site-directed mutagenesis was performed on the GluCl β subunit using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA), and the mutations were confirmed by sequencing.

2.2. Fluorescent protein-tagged constructs

Enhanced green fluorescent protein (EGFP), yellow fluorescent protein (EYFP), and cyan fluorescent protein (ECFP) were obtained from ClonTech. The PCR primers 5′-TCCACCGGGCCGGCAAATGGTGAGCAAGGGC-3′ and 5′-GTCGCGGCGCCGGCGCTTGTACAGCTCGTCCA-3′ were used to PCR amplify EYFP or EGFP, and the resultant PCR product was ligated into the *Ngo*MIV site of GluCl α, which positions the tag immediately after the arginine at residue 408, the 49th out of 73 residues in the M3–M4 loop. The PCR primers 5′-CACCGGTCTTCGAAATGGTGAGCAAGGGC-3′ and 5′-TCGCGGCCTTCGAACTTGTACAGCTCGTCC-3′ were used to PCR amplify ECFP, and the resultant PCR product was ligated into the *Csp*45I site of GluCl β, which positions the tag immediately after the phenylalanine at residue 373, the 43rd out of 69 residues in the M3–M4 loop.

2.3. Expression of GluCl cRNAs

The cDNAs were linearized by *Not*I. All cRNAs were transcribed in vitro using the T7 mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA) as appropriate. RNAs were then quantitated by RiboGreen™ RNA Quantitation kit (Molecular Probes, Eugene, OR, USA). Stages V–VI *Xenopus laevis* oocytes were harvested and injected with 50 nl/oocyte of a mixture containing 25–375 pg per subunit of cRNAs for WT, mutant and XFP constructs, in equimolar amounts of α and β. The size of the XFP coding region is ~0.7 kb and that of the GluCl subunits is ~1.4 kb, thus 1.5 times greater mass of an XFP-tagged subunit cRNAs was injected compared with WT subunits. After injection, oocytes were incubated at 18°C in ND-

*Corresponding author. Fax: (1)-626-564 8709.
E-mail address: lester@caltech.edu (H.A. Lester).

PII: S0014-5793(02)03245-3

96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 5 mM HEPES, pH 7.5, osmolarity 230 mOsm) supplemented with 50 μg/ml gentamicin, 2.5 mM pyruvate and 0.6 mM theophylline.

2.4. Electrophysiology

Recordings were carried out 24–36 h after injection. Membrane potential was held at −60 mV in Ca^{2+}-free ND-96 with two electrodes (filled with 3 M KCl, resistance 0.5–3 MΩ) using a GeneClamp 500 circuit and a Digidata 1200 digitizer from Axon Instruments (Union City, CA, USA) interfaced with an IBM-compatible PC running pCLAMP 8.0 software from Axon. Drugs were applied with a dead time of 1 s. Typical drug application times for glutamate and IVM were 10 s and 30 s, respectively. Experiments were conducted at room temperature (22°C). IVM was stored frozen as a 10 mM stock solution in dimethyl sulfoxide for up to 2 weeks. When dissolved into the perfusion solution, the final concentration of dimethyl sulfoxide was no more than 0.1%. Glutamate was also prepared from stock solution at a concentration of 100 mM (in water).

2.5. Data analysis

The glutamate dose–response relations were measured by applying a series of glutamate concentrations to each oocyte. Because IVM responses did not reverse on the time scale of the experiments, only a single concentration was applied to each oocyte. This response was normalized to the amplitude of the response to 5 μM IVM in oocytes (a saturating concentration) of the same batch. Glutamate and IVM responses to various drug concentrations were fitted to the Hill equation by a non-linear routine, $I/I_{max} = 1/(1+(EC_{50}/[A])^n)$, where I is agonist-induced current at concentration $[A]$, I_{max} is the maximum current, EC_{50} is the concentration inducing half-maximum response, and n is the Hill coefficient. Statistical analyses were performed using unpaired two-population Student's t-test (as appropriate), with a significance level of $P < 0.05$. All data were presented as mean ± S.D.

2.6. Drugs and restriction endonucleases

Glutamate and IVM were purchased from Sigma (St. Louis, MO, USA); Csp45I was purchased from Promega (Madison, WI, USA); and the other restriction endonucleases were purchased from New England Biolabs (Beverly, MA, USA).

3. Results and discussion

3.1. Mutations in the ligand-binding domain and M1 of GluCl β

To eliminate sensitivity to glutamate, we mutated the GluCl

```
c GluCl β  ----------------------------------MTTPSSFSILLLLLLMPVVTNGEYSMQSEQEILNALLKN-YD  41
c GluCl α  MATWIVGKLIIASLILGIQAQQARTKSQDIFEDDNDNGTTTLESLARLTSPIHIPIEQPQTSDSKILAHLFTSGYD  76
h GlyR α1  ------------------------------------------------ARSATKPMSPSDFLDKLMGRT--SGYD  25
m GABA β2  -------------------------MWRVRKRGYFGIWSFPLIIAAVCAQSVNDPSNMSLVKETVDRLL--KGYD  48
h ACh  α1  -----MEPWPLLLLFSLCSAGLVLGSEHETR-----LVAKLFKDYSSVVRPVEDHRQVVEVTVGLQLIQLINVDEV  66
h 5-HT3A   MLLWVQQALLALLLPTLLAQGEARRSRNTTRPALLRLSDYLLTNYRKGVRPVRDWRKPTTVSIDVIVYAILNVDEK  76

c GluCl β  MRVRPPPANSSTEGAVNVRVNIMIRMLSKIDVVNMEYSIQLTFREQWIDPRLAYENLGFYNPPAFLTVPHVKKSLWI 118
c GluCl α  FRVRPPTDNG---GPVVVSVNMLLRTISKIDVVNMEYSAQLTLRESWIDKRLSYGVKGDGQPDFVILT--VGHQIWM 148
h GlyR α1  ARIRPNFKGP----PVNVSCNIFINSFGSIAETTMDYRVNIFLRQQWNDPRLAYNEYPD---DSLDLDPSMLDSIWK  95
m GABA β2  IRLRPDFGGP----PVAVGMNIDIASIDMVSEVNMDYTLTMYFQQAWRDKRLSYNVIPL---N-LTLDNRVADQLWV 117
h ACh  α1  NQIVTTN--------VRLKQGDMVDLPRPSCVTLGVPLFSHLQNEQWVDYNLKWNPDDY---GGVKKIHIPSEKIWR 132
h 5-HT3A   NQVLTTY--------IWYRQ-------------------------YWTDEFLQWNPEDF---DNITKLSIPTDSIWV 117

c GluCl β  PDTFFPTEKAAHRHLIDMENMFLRIYPDGKILYSSRISLTSSCPMRLQLYPLDYQSCNFDLVSYAHTMNDIMYE-WD 194
c GluCl α  PDTFFPNEKQAYKHTIDKPNVLIRIHNDGTVLYSVRISLVLSCPMYLQYYPMDVQQCSIDLASYAYTTKDIEYL-WK 224
h GlyR α1  PDLFFANEKGAHFHEITTDNKLLRISRNGNVLYSIRITLTLACPMDLKNFPMDVQTCIMQLESFGYTMNDLIFE-WQ 171
m GABA β2  PDTYFLNDKKSFVHGVTVKNRMIRLHPDGTVLYGLRITTTAACMMDLRRYPLDEQNCTLEIESYGYTTDDIEFY-WR 193
h ACh  α1  PDLVLYNNADGDFAIVKFTKVLLQ--YTGHITWTPPAIFKSYCEIIVTHFPFDEQNCSMKLGTWTYDGSVVAIN--- 204
h 5-HT3A   PDILINEFVDVGKS-PNIPYVYIR--HQGEVQNYKPLQVVTACSLDIYNFPFDVQNCSLTFTSWLHTIQDINISLWR 191

c GluCl β  PSTPVQLKPGVGSDLPNFILKNYTTNADC--T HTNTGSYGCLRMQLLFKRQFSYYLVQLYAPTTMIVIVSWVSFWI 269
c GluCl α  EHSPLQLKVGLSSSLPSFQLTN-TSTTYC--TSVTNTGIYSCLKTTIQLKREFSEYLLQLVIPSCMLVIVSWVSFWF 298
h GlyR α1  -EQGA-VQVADGLTLPQFILKEEKDLRYC--TKHYNTGKFTCIEARFHLERQMGYYLIQMYIPSLLIVILSWISFWI 244
m GABA β2  GDDNA-VTGVTKIELPQFSIVDYKLITKK--VV-FSTGSYPRLSLSFKLKRNIGYFILQTYMPSILITILSWVSFWI 267
h ACh  α1  -PESDQPDLSNFMESGEWVIKESRGWKHSVTYSCCPDTPYLDITYHFVMQRLPLYFIVNVIIPCLLFSFLTGLVFYL 280
h 5-HT3A   LPEKVKSDRSVFMNQGEWELLGVLPYFRE--FSMESSNYYAEMKFYVVIRRRPLFYVVSLLLPSIFLMVMDIVGFYL 266
                                                                                  M1
```

Completely conserved, partially conserved, partially conserved
Basic in GluCl β and GluCl α but not in the other receptors
Crucial for binding
Loop C, not glycine
Cys
See text

Fig. 1. GluCl sequences in the extracellular ligand-binding domain and M1, compared with other nAChR superfamily members. The *C. elegans* GluCl β subunit and α subunit (c GluCl β; c GluCl α; GenBank accession numbers U14525 and U14524) were aligned with each other and the human glycine receptor α_1 subunit (h GlyR α_1; Swiss-Prot P23415), mouse GABA receptor β_2 subunit (m GABA β_2; Swiss-Prot P15432), human α_1 nAChR subunit (h ACh α_1; Swiss-Prot P02708) and human 5-HT_{3A} subunit (h 5-HT_{3A}; Swiss-Prot P46098), using MULTALIN (http://pbil.ibcp.fr/cgi-bin/align_multalin.pl). The first two Cys residues define the signature loop common to all nAChR superfamily subunits, and the second two Cys define loop C in nAChR. Sixteen highlighted residues on GluCl β were mutated to alanine. At Y182 and Y232 (yellow residues), tyrosine was also mutated to phenylalanine and tryptophan. Y182 aligns with W149 at nAChR, the site of a cation–π interaction with the ligand. T229 and Y232 (yellow residues) align with the crucial nAChR Y190 and Y198, respectively. I269 aligns with I244 in glycine receptor. Six additional mutations to alanine in loop C were performed, where the residue is not glycine. The other six residues are basic in GluCl α and GluCl β but not in the other receptors, suggesting they may interact with the acidic group of glutamate. These residues were also mutated to alanine.

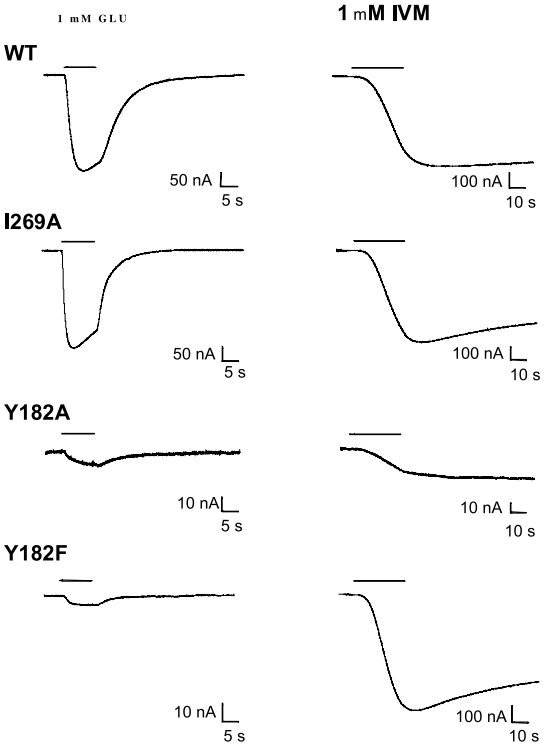

Fig. 2. Electrophysiological characterization of WT and three mutated GluCl constructs. Left-hand column, responses to 1 mM glutamate (GLU); right-hand column, responses to 1 μM IVM; the latter are slower than the glutamate responses and irreversible on the time scale of the experiments. The top row shows responses in an oocyte injected with GluCl αβ(WT) from *C. elegans*. Oocytes injected with GluCl αβ(I269A) responded well to both glutamate and IVM. The αβ(Y182A) mutant GluCl abolished both glutamate and IVM responses. The bottom row shows glutamate sensitivity was abolished but IVM sensitivity was retained in GluCl αβ(Y182F). For each mutant, the two traces shown were obtained from one oocyte. Horizontal line over the traces indicates the application time of agonists. *Xenopus* oocytes were injected with 25 pg of both WT α RNA and WT (or mutated) β RNA.

β subunit, because this subunit is required for glutamate sensitivity [11]. GluCl channels are members of the nicotinic acetylcholine receptor (nAChR) superfamily [10]. Based on structure–function studies of the glycine receptor [19] and the crystal structure of the ACh binding protein [14,20], we identified 16 residues likely to be involved in the response to glutamate but not to IVM.

Fig. 1 shows the alignment between GluCls and four other nAChR family members. Residues in red type are completely conserved; green and blue type denote partial conservation. The first two Cys residues define the 'signature loop' common to all nAChR superfamily subunits, and the second two Cys define loop C (nAChR terminology). Y182 in the GluCl β subunit aligns with W149 at nAChR, the site of a cation–π interaction with the ligand [20,21]. T229 and Y232 align with the crucial nAChR Y190 and Y198, respectively. The glycine receptor is the closest homolog of GluCls in vertebrates. Relevant site-directed mutagenesis experiments on human glycine receptors indicate that I244A eliminates glycine sensitivity but retains IVM sensitivity [19]. I269 at the GluCl β subunit aligns with this position. These four residues were mutated in the first round (highlighted in yellow, figure legend, Fig. 1). The mutations Y182A and Y232A abolished both glutamate and IVM responses; at these residues, we then performed four 'milder' mutations (Y→F, W). We have not succeeded in generating a cDNA for Y232F.

We also generated and analyzed six additional X→A mutations in loop C [22,23], where X is not glycine (highlighted in green, figure legend, Fig. 1). They are T224A, S225A, H226A, T227A, N228A and S231A. Red residues in Fig. 1 (R66, K70, R131, K202, K236 and K242) are basic in GluCl α and GluCl β, but not in the other receptors, suggesting that they may interact with the acidic group of glutamate; we mutated these residues to alanine as well.

3.2. Glutamate and IVM responses of WT GluCl receptors

Heteromeric receptors expressed robustly after injections of 25 pg each of cRNA for the GluCl α and β subunits (termed WT). Response amplitudes were 304 ± 18 nA ($n = 33$) to 1 mM glutamate and 675 ± 26 nA to 1 μM IVM ($n = 26$). Fig. 2 (top panel) presents an example of WT responses to the agonists. 1 mM glutamate induced a rapid and reversible current (348 nA) while 1 μM IVM induced a slow and irreversible current (596 nA). The EC_{50} and the Hill coefficient for WT are 0.48 ± 0.08 μM and 1.76 ± 0.31 for IVM and 0.32 ± 0.05 mM and 1.69 ± 0.07 ($n = 6$) for glutamate, respectively.

As expected from previous data [11], responses were much smaller for equivalent injections of individual cRNAs, which presumably encoded homomeric receptors. When 25 pg cRNA of GluCl α or GluCl β was injected, the average responses to 1 μM IVM or 1 mM glutamate were 24 ± 6 nA ($n = 6$) or 79 ± 8 nA ($n = 10$), respectively, some 10-fold smaller than the heteromeric responses. Responses were considerably larger with injection of 250 pg cRNA for the individual subunits. Oocytes injected with 250 pg GluCl α cRNA displayed an IVM dose–response relation with an EC_{50} of 0.52 ± 0.06 μM, a Hill coefficient of 2.24 ± 0.35, and maximal responses of 1.68 ± 0.25 μA ($n = 8$); there was little or no response to glutamate. For injections of 250 pg GluCl β cRNA, the responses

Table 1
Summary of glutamate and IVM effects on WT and mutant GluCls

GluCl	Glutamate $I/I_{\alpha\beta(WT)}$	IVM $I/I_{\alpha\beta(WT)}$
α(WT)	0	0.15 ± 0.04
β(WT)	0.10 ± 0.02	0
αβ(WT)	1	1
αβ(Y182A)	0.01 ± 0.01	0.05 ± 0.02
αβ(T229A)	0.98 ± 0.17	0.85 ± 0.29
αβ(Y232A)	0.02 ± 0.01	0.12 ± 0.05
αβ(I269A)	1.36 ± 0.20	1.29 ± 0.48
αβ(Y182F)	0.16 ± 0.03	1.06 ± 0.27
αβ(Y182W)	0.14 ± 0.05	0.32 ± 0.14
αβ(Y232F)	N/A	N/A
αβ(Y232W)	0.14 ± 0.06	0.29 ± 0.16
αβ(T224A)	0.739	0.352
αβ(S225A)	2.23	0.87
αβ(H226A)	1.10	0.91
αβ(T227A)	0.85	0.73
αβ(N228A)	0.93	1.20
αβ(S231A)	0.76	1.40
αβ(R66A)	0.15	0.45
αβ(K70A)	0.48	0.58
αβ(R131A)	0.85	0.51
αβ(K202A)	1.17	0.55
αβ(R236A)	0.02	0.21
αβ(K242A)	0.13 ± 0.06	0.56 ± 0.15

All mutants were in the β subunit. All values were averaged from at least five oocytes. For those mutants examined in more than two batches of oocytes, data are presented as mean ± S.E.M.

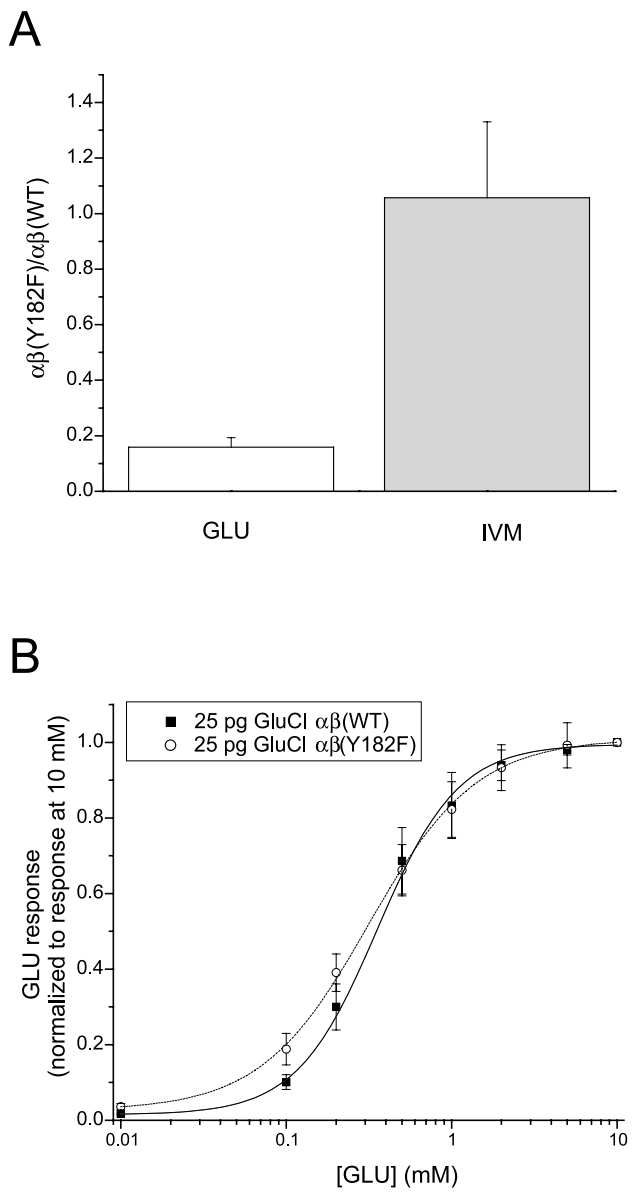

Fig. 3. GluCl αβ(Y182F) eliminates glutamate sensitivity and retains IVM sensitivity. A: GluCl αβ(Y182F) reduces the glutamate response to 15.8% of WT levels but hardly changes IVM responses (106% of WT values). The values were pooled from 78 oocytes of six batches, injected with 25 pg to 250 pg of cRNA for each subunit. Error bars are S.E.M. B: Normalized glutamate dose–response relations for WT and GluCl αβ (Y182F). For GluCl αβ (Y182F) (open symbols, dashed line), the curve represents an EC_{50} of 0.32 mM and an n_H of 1.33 ($n=5$), similar to those of the WT GluCl αβ (closed symbols, solid line) (0.36 mM; 1.78, $n=6$).

to glutamate displayed an EC_{50} of 0.24 ± 0.01 mM, a Hill coefficient of 1.56 ± 0.28 ($n=5$), and maximal response of 0.90 ± 0.13 μA ($n=15$); responses to IVM were small or absent. The homomeric receptors showed more rapid kinetics (activation, desensitization and deactivation) than the heteromeric responses; we did not study this phenomenon systematically.

3.3. Glutamate and IVM responses at mutant receptors

In our first round of mutagenesis experiments, four constructs were tested in oocytes. GluCl αβ(T229A) and GluCl αβ(I269A) responded like WT: no significant difference was found ($P>0.05$, $n=20$). Fig. 2 (second row) shows the currents induced from an oocyte injected with GluCl αβ(I269A), 360 nA and 710 nA for glutamate and IVM, respectively. On the other hand, GluCl αβ(Y182A) and GluCl αβ(Y232A) abolished both glutamate and IVM sensitivities (Fig. 2, third row).

Fifteen additional mutants were examined in the second round (summarized in Table 1). Mutations to Ala (X→A) at H226, T227, N228, or S231 did not affect functional expression ($P>0.05$, $n=6$); mutations of T224, R131 or K202, did not affect glutamate response but modestly reduced (two- to three-fold) the IVM response; and mutations at R66, K70, R236 and K242 decreased both glutamate and IVM responses. Mutations (Y→W) at Y182 and Y232 also destroyed both glutamate and IVM sensitivities. GluCl αβ(S225A) significantly increased the glutamate response to 223% of WT levels ($P<0.05$, $n=6$). Thus, none of these mutations achieved the desired results.

3.4. GluCl αβ(Y182F) eliminates glutamate responses while retaining IVM responses

At position Y182 of the GluCl β subunit, three mutations were constructed (Y→A, W, F). The mutants GluCl αβ(Y182A) and GluCl αβ(Y182W) abolished the response of both glutamate and IVM. However, GluCl αβ(Y182F) meets our requirement. An example is shown in Fig. 2 (bottom row). 1 mM glutamate induced a tiny current (~5 nA) in the oocyte that expressed GluCl αβ(Y182F), while 1 μM IVM induced a current of 827 nA. On average, Y182F, when coexpressed with WT GluCl α, reduced the glutamate response to 15.8 ± 3.4% of WT levels but hardly changed IVM responses (106 ± 27%) ($n=78$) (Fig. 3A). For GluCl αβ(Y182F), the EC_{50} and Hill coefficient for glutamate is 0.32 ± 0.05 mM and 1.33 ± 0.25 ($n=5$), respectively, similar to those of WT (0.36 ± 0.04 mM, 1.78 ± 0.22, $n=6$) (Fig. 3B), indicating that the mutant decreases the efficacy of glutamate, but not the potency.

Tyr-182 aligns with nicotinic receptor residue Trp-149, which plays an important role in a cation–π interaction with the quaternary ammonium moiety of acetylcholine [20,21]. We conclude that this residue also plays an important role in the binding or response to both glutamate and IVM in the GluCl β subunit. The interaction with both ligands is abolished by mutating the Tyr to Ala or to Trp, but only the interaction with glutamate is weakened by mutations to Phe. The available data did not allow a decision about the nature of the moiety that interacts with Tyr182, or the nature of the interaction.

3.5. Functional expression of fluorescent protein-tagged constructs

Three fluorescent proteins (EGFP, EYFP, ECFP; XFP) were separately inserted into the M3–M4 loop of the GluCl α, β and β(Y182F). Firstly, functional expressions of GluCl α(GFP), GluCl α(YFP), GluCl β(CFP) and GluCl β(Y182F)(CFP) were expressed as homomers in oocytes and compared with GluCl α, β and β(Y182F), respectively. No significant difference was observed ($P>0.05$, $n>6$, data not shown). Then heteromers with XFP tags in only the α or β subunit constructs were examined. Data for one of these constructs are given in Fig. 4A. No significant difference was found between GluCl αβ(Y182F) and GluCl

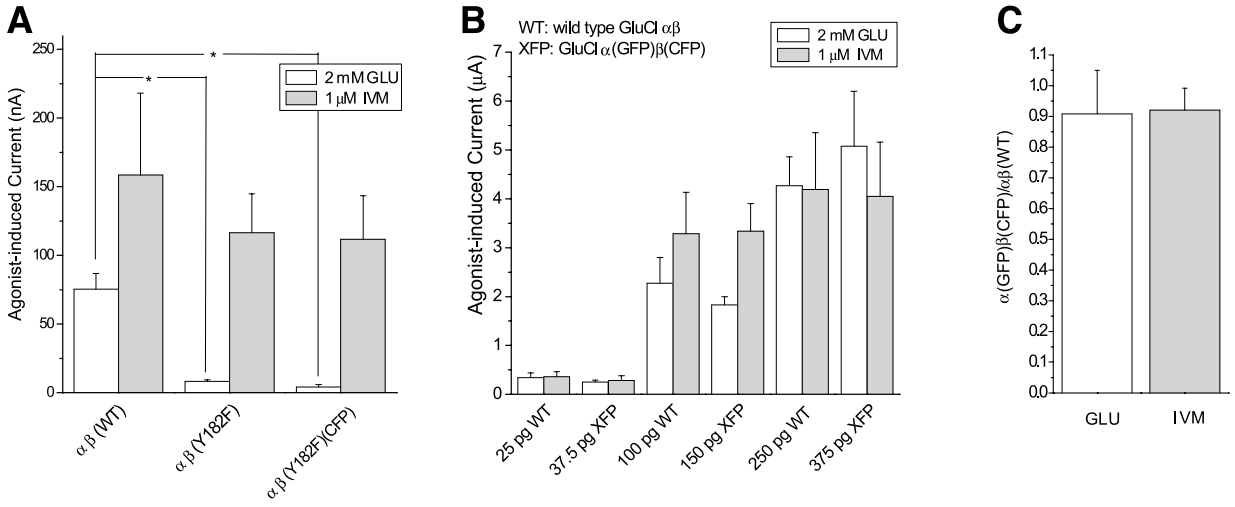

Fig. 4. Functional expression of fluorescent protein-tagged constructs. A: Comparison among GluCl αβ, αβ(Y182F) and αβ(Y182F)(CFP). No significant difference is found between the functional expression of β(Y182F) and β(Y182F)(CFP). All values were averaged from six to nine oocytes. Error bars are S.E.M. and asterisks represent significant difference between two groups ($P < 0.05$). Oocytes were coinjected with 25 pg WT α RNA and 25 pg WT β, β(Y182F) or 37.5 pg β(Y182F)(CFP) RNA, respectively. B: Bar plots compare WT and GluCl α(GFP)β(CFP) (termed XFP). There is no significant difference ($P > 0.05$) between the responses in oocytes injected with equal molar concentration of WT and XFP RNA. C: Grand average from 30 oocytes injected with three concentrations (25 pg, 100 pg and 250 pg for each subunit of WT; 37.5 pg, 150 pg and 375 pg for each subunit of XFP). The glutamate and IVM responses of XFP are 90.8% and 92.0% of WT levels, respectively.

αβ(Y182F)(CFP) ($P > 0.05$, $n = 9$). Like GluCl αβ(Y182F), GluCl αβ(Y182F)(CFP) abolished the glutamate response to 10.9% of the WT level and almost kept the IVM response (84.6% of the WT level) ($n = 7$). Finally, doubly XFP-tagged constructs were expressed to the oocytes. Three different molar concentrations of RNA were compared with WT and XFPs. There is no significant difference ($P > 0.05$, $n > 6$) between the responses in oocytes injected with equal molar concentrations of WT and XFP RNA (Fig. 4B). The grand averaged data from 30 oocytes showed that XFP hardly changed the response to both glutamate and IVM (90.8 ± 14.2% and 92.0 ± 7.1% of WT level, respectively) (Fig. 4C). The re-engineered GluCl channel, without glutamate sensitivity but with a fluorescent tag, may be more useful in GluCl silencing strategies.

3.6. Critique of the GluCl/IVM strategy

Channel-based silencing strategies could mimic either the endogenous inhibition arising from activation of K^+ channels (for instance, $GABA_B$ receptors coupled to GIRK channels) or that arising from activation of Cl^- channels (for instance, $GABA_A$ receptors). Most strategies employ K^+ channels [1–8]. K^+ channels have the advantage that they can be chosen, or modified, to display various voltage-dependent and rectifying characteristics. K^+ channels have the disadvantage that sustained K^+ efflux (or Na^+ entry) causes apoptosis under some circumstances [5].

The GluCl/IVM strategy [9] is, to our knowledge, unique among artificial silencing strategies because it employs Cl^- channels. In most cells, intracellular [Cl^-] is on the order of a few mM; as a result, if G_{Cl} rises, intracellular Cl^- is expected to be driven by the K^+ gradient. Therefore a high Cl^- conductance is expected to clamp the cell near E_K, or slightly hyperpolarized from the normal resting potential, and to cause only minor changes in intracellular Cl^-. One possible disadvantage to this strategy involves such neurons as dorsal root ganglia neurons and many immature central nervous system neurons, whose complement of transporters leads to a relatively high intracellular Cl^- and therefore a depolarized E_{Cl}. In such a cell, the GluCl/IVM strategy might cause E_{Cl} and E_K to approach each other, possibly leading to KCl loss and to cell shrinkage. It is encouraging that in previous experiments on dorsal root ganglion cells, a heterologously expressed, continuously active Cl^- conductance did cause a hyperpolarizing shift in E_{Cl}, but neither shrank nor killed the cells [24].

Acknowledgements: We thank Kira Kostenko and Carrie Shilyansky for help with the oocytes and members of our research group for helpful discussion. This research was supported by a grant from the National Institutes of Health (NS-11756) and by the William T. Gimbel Discovery Fund in Neuroscience.

References

[1] Johns, D.C., Marx, R., Mains, R.E., O'Rourke, B. and Marban, E. (1999) J. Neurosci. 19, 1691–1697.
[2] White, B., Osterwalder, T. and Keshishian, H. (2001) Curr. Biol. 11, R1041–1053.
[3] White, B.H., Osterwalder, T.P., Yoon, K.S., Joiner, W.J., Whim, M.D., Kaczmarek, L.K. and Keshishian, H. (2001) Neuron 31, 699–711.
[4] Falk, T., Kilani, R.K., Yool, A.J. and Sherman, S.J. (2001) Gene Ther. 8, 1372–1379.
[5] Nadeau, H., McKinney, S., Anderson, D.J. and Lester, H.A. (2000) J. Neurophysiol. 84, 1062–1075.
[6] Scearce-Levie, K., Coward, P., Redfern, C.H. and Conklin, B.R. (2001) Trends Pharmacol. Sci. 22, 414–420.
[7] Sutherland, M.L., Williams, S.H., Abedi, R., Overbeek, P.A., Pfaffinger, P.J. and Noebels, J.L. (1999) Proc. Natl. Acad. Sci. USA 96, 2451–2455.
[8] Lechner, H.A., Lein, E.S. and Callaway, E.M. (2002) J. Neurosci. 22, 5287–5290.
[9] Slimko, E., McKinney, S., Anderson, D., Davidson, N. and Lester, H.A. (2002) J. Neurosci. 22, 7373–7379.
[10] Xue, H. (1998) J. Mol. Evol. 47, 323–333.
[11] Cully, D.F., Vassilatis, D.K., Liu, K.K., Paress, P.S., Van der Ploeg, L.H., Schaeffer, J.M. and Arena, J.P. (1994) Nature 371, 707–711.

[12] Corringer, P.J., Le Novere, N. and Changeux, J.P. (2000) Annu. Rev. Pharmacol. Toxicol. 40, 431–458.
[13] Etter, A., Cully, D.F., Liu, K.K., Reiss, B., Vassilatis, D.K., Schaeffer, J.M. and Arena, J.P. (1999) J. Neurochem. 72, 318–326.
[14] Dougherty, D.A. and Lester, H.A. (2001) Nature 411, 252–253, 255.
[15] Han, N.L., Haddrill, J.L. and Lynch, J.W. (2001) J. Neurochem. 79, 636–647.
[16] Curtis, L., Chiodini, F., Spang, J.E., Bertrand, S., Patt, J.T., Westera, G. and Bertrand, D. (2000) Eur. J. Pharmacol. 393, 155–163.
[17] Pedersen, S.E., Dreyer, E.B. and Cohen, J.B. (1986) J. Biol. Chem. 261, 13735–13743.
[18] Gensler, S., Sander, A., Korngreen, A., Traina, G., Giese, G. and Witzemann, V. (2001) Eur. J. Biochem. 268, 2209–2217.
[19] Shan, Q., Haddrill, J.L. and Lynch, J.W. (2001) J. Biol. Chem. 276, 12556–12564.
[20] Brejc, K., van Dijk, W.J., Klaassen, R.V., Schuurmans, M., van der Oost, J., Smit, A.B. and Sixma, T.K. (2001) Nature 411, 269–276.
[21] Zhong, W., Gallivan, J.P., Zhang, Y., Li, L., Lester, H.A. and Dougherty, D.A. (1998) Proc. Natl. Acad. Sci. USA 95, 12088–12093.
[22] Vandenberg, R.J., Rajendra, S., French, C.R., Barry, P.H. and Schofield, P.R. (1993) Mol. Pharmacol. 44, 198–203.
[23] Rajendra, S., Vandenberg, R.J., Pierce, K.D., Cunningham, A.M., French, P.W., Barry, P.H. and Schofield, P.R. (1995) EMBO J. 14, 2987–2998.
[24] Staley, K., Smith, R., Schaack, J., Wilcox, C. and Jentsch, T.J. (1996) Neuron 17, 543–551.