FEBS Letters 426 (1998) 174–178 FEBS 20100

Topological localization of cysteine 74 in the GABA transporter, GAT1, and its importance in ion binding and permeation

Nam Yu[1], Yongwei Cao[2], Sela Mager[3], Henry A. Lester*

Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125, USA

Received 21 January 1998; revised version received 9 March 1998

Abstract ***Xenopus*** **oocytes expressing the GABA transporter GAT1 were exposed to membrane-impermeant sulfhydryl reagents, resulting in decreased GABA transport current, decreased capacitive charge movements, and increased Na^+ and Li^+ leakage currents. Mutation of cysteine 74 to alanine (C74A) eliminated these effects. The W68S and W68L mutations significantly increased and decreased the transporter's sensitivity, respectively, to sulfhydryl reagents. At each of the positions 73 through 76, cysteine residues were accessible to external MTSET. These findings, together with recent evidence placing the HD2–HD3 loop on the extracellular side, suggest that the HD2 region does not traverse the membrane.**

Key words: Neurotransmitter transporter; *Xenopus* oocyte; Cysteine reagent; Membrane topology; Electrophysiology

1. Introduction

The GABA transporter GAT1 [1] functions with the stoichiometry of one GABA molecule, two Na^+ ions, and one Cl^- ion [2–4]. Hydropathy analysis of GAT1 and related neurotransmitter transporters reveals 12 putative hydrophobic domains; in the initial structural hypothesis, all these domains completely traversed the membrane as α-helices [1]. This model places both the amino- and carboxyl-terminal ends in the intracellular compartment and predicts the presence of a large extracellular hydrophilic loop containing three N-linked glycosylation sites between HD3 and HD4 [1].

Topology of Na^+-, Cl^--coupled transporters remains an unsolved problem [5–9]. However, cysteine accessibility experiments, in conjunction with electrophysiological measurements, have given valuable information about the topology of ion channels [10–14]. We have therefore performed similar studies on GAT1.

2. Materials and methods

2.1. Heterologous expression in oocytes

cRNAs of wild-type (WT) W68L, W68S, and C74A GAT1 were transcribed in vitro from linearized pAMV-PA [4,15] plasmid constructs containing the respective cDNA clones and optimized for oocyte expression. The cysteine mutants L73C/C74A, G75C/C74A, and K76C/C74A were constructed in a two-stage PCR procedure. The first PCR reaction used WT GAT1 as the template in two separate tubes with the following primers: tube a, sense T7 promoter with antisense mutagenic primer; tube b, antisense T3 promoter with sense mutagenic primer. The partial-length fragments from tubes a and b were gel purified. The second PCR was performed using the two fragments from the first step as the template and only the T7 and T3 primers. RNA was transcribed directly from the resulting, full-length product using T7 RNA polymerase and gave reduced but robust levels of expression.

Stage V and VI oocytes were isolated [16] and injected with 10–20 ng of mRNA in 25 nl of water. Injected oocytes were incubated 4–7 days at 19°C before recordings were made.

2.2. Electrophysiology

Recordings were made at room temperature (21–22°C) using the two-electrode voltage-clamp technique [16]. Data acquisition and analysis were performed using the pCLAMP program suite (Axon Instruments, Foster City, CA).

The standard Na^+ Ringer medium contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, and 1 mM $CaCl_2$. *N*-methyl-D-glucamine ($NMDG^+$) and Li^+ Ringer solutions were prepared by equimolar substitution of NaCl by NMDG-Cl or LiCl, respectively.

2.3. Sulfhydryl modification by methanethiosulfonate (MTS) derivatives

MTSEA (MTS-ethylammonium), MTSET (MTS-ethyltrimethylammonium), and MTSES (MTS-ethylsulfonate) (Toronto Research Chemical Co., Toronto, Canada) were used for irreversible, covalent modification of cysteine residues. After initial electrophysiological characterization, oocytes were removed from the voltage-clamp chamber and incubated in Ringer solution containing the indicated concentrations of the MTS reagent. After incubation, the oocytes were washed and voltage-clamped a second time; these recordings were compared with pre-incubation records to examine the effects of sulfhydryl modification.

MTS compounds were prepared as a 1.0 M solution in H_2O and kept on ice. This stock solution was diluted in the appropriate buffer solution at room temperature just before exposure to the oocyte.

3. Results

3.1. Reactivity of WT and mutant GAT1 to MTS reagents

In *Xenopus* oocytes expressing WT GAT1, GABA-induced transport-associated currents were reduced after incubation with MTSET, suggesting chemical modification in a functionally sensitive region(s) of the protein (Fig. 1). As described below, inhibition was also observed after exposure to MTSEA and MTSET (Fig. 2). The original topology model [1] places three cysteine residues on the extracellular side. Data obtained from dopamine and serotonin transporters reveal that two of these three cysteine residues are critical for plasma membrane targeting [17] and are thought to form a disulfide bridge [8], which is insensitive to MTS reagents. This leaves cysteine 74 as the prime target for modification by membrane-impermeant sulfhydryl reagents.

*Corresponding author. Fax: +1 (626) 564-8709.
E-mail: lester@caltech.edu

[1]Present address: Harvard Medical School, Boston, MA 02115, USA.

[2]Present address: Monsanto Company, AA5I, St. Louis, MO 63198, USA.

[3]Present address: Department of Physiology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7545, USA.

PII S0014-5793(98)00333-0

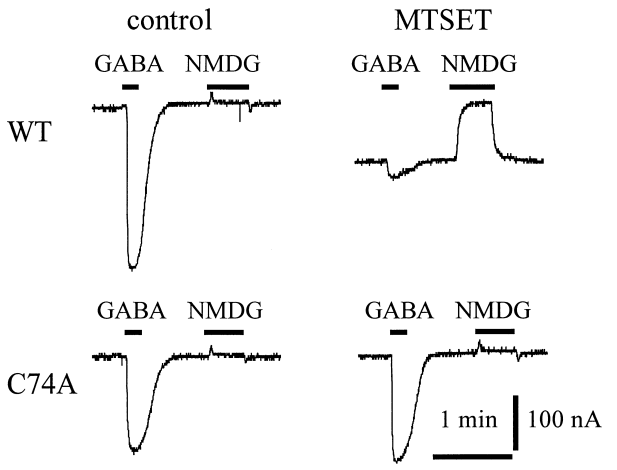

Fig. 1. Effect of MTSET treatment on transport-associated and leakage currents of GAT1. Oocytes expressing WT (upper traces) or C74A (lower traces) mutant GAT1 were analyzed before (left column) and after incubation in MTSET (right column). The holding potential was −40 mV. MTSET (3.75 mM) was applied for 30 min. GABA and $NMDG^+$ Ringer solutions were perfused in the oocyte chamber for the times indicated by the bars. GABA application produced an inward transport-associated current. Removal of Na^+ ($NMDG^+$ substitution) produced an outward deflection, corresponding to the inhibition of an inward current.

We mutated this cysteine residue to alanine (C74A) and tested the mutant transporter's sensitivity to sulfhydryl reagents. Before exposure to sulfhydryl reagents, the mutant transporter displayed no differences from the WT in electrophysiological properties (transport-associated currents in the presence of GABA and charge movements in the absence of GABA).

C74A was still inhibited by MTSEA, though less than WT GAT1 (Fig. 2A). However, MTSET did not inhibit C74A activity (Fig. 2B). Another MTS reagent, the negatively charged MTSES, displayed a pattern of inhibition similarly to that of MTSET (Fig. 2C). A possible explanation is that MTSEA is attacking cysteine residues located inside the cell, consistent with reports that MTSEA can permeate the membrane while MTSET and MTSES can not [18,19]. The specific MTSET reaction with C74 provided a convenient approach to study this region of the protein from both structural and functional aspects.

Tryptophan 68 in HD1 partially governs ion binding and permeation at GAT1 [20]. The W68S mutant reacted much faster with MTSET than did WT GAT1 (Fig. 2B). The W68S/C74A double mutant was unaffected by MTSET treatment (data not shown), indicating that the increased sensitivity of W68S was not due to exposure of a new cysteine. The W68L mutant, on the other hand, was significantly less sensitive ($<20\%$ decrement after 10 min in 2.0 mM MTSET, data not shown).

3.2. MTSET also decreases charge movements

In addition to eliminating transport-associated current, C74 modification by MTSET resulted in decreases in transporter-dependent transient currents induced by voltage-jump relaxations (Fig. 3). These transient currents are capacitive charge movements carried by Na^+ ions which bind to and dissociate from the transporter; the Na^+ binding step is thought to occur before GABA binding and translocation [4,20]. The charge movements, obtained by integrating the transient part of the traces [4], were decreased by $>90\%$ (three oocytes) under our conditions (Fig. 3C). In place of the transient currents, we observed large currents that were sustained for the duration of the test pulse (see Section 3.3). MTSES incubation also abolished the transient currents (data not shown).

The C74A mutant also displayed capacitive charge movements, as expected from the functional similarities between C74A and WT GAT1 (Fig. 3). However, MTSET treatment produced virtually no change in the charge movements, as measured by integrating the transient phase ($<10\%$ decrement under the same conditions; see Fig. 3A, C).

3.3. MTSET induces large leakage currents

When MTSET-treated oocytes expressing the WT transporter were exposed to Ringer solution in which Na^+ was replaced by $NMDG^+$, a large, steady-state outward current deflection was evoked (Fig. 1). This deflection returned the current nearly to the baseline level before MTSET treatment. This pattern indicates that the deflection was caused by inhibition of a novel inward current, presumably from increased Na^+ leakage through MTSET-modified transporters, rather than by induction of outward current. Leakage current produced by Li^+ also increased after incubation, though to a lesser extent than did Na^+ leakage current (data not shown). Current voltage analysis revealed that the Na^+ leakage cur-

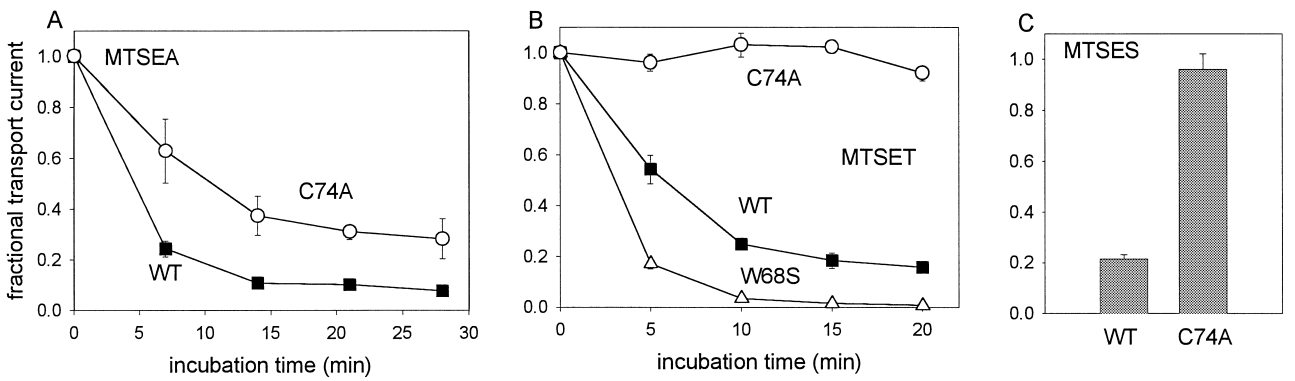

Fig. 2. Reactivity of WT, C74A, and W68S GAT1 to cysteine reagents. A: Inactivation of transport-associated current following treatment by MTSEA (750 μM) is shown for the incubation times indicated. Points represent the mean ± S.D. of triplicate sets. B: Exposure of WT, W68S, and C74A GAT1 to MTSET (2.0 mM). All oocytes represented were from the same batch. Points represent the mean ± S.D. of triplicate sets. C: WT- and C74A-expressing oocytes were incubated with MTSES (3.75 mM, 30 min). Bars represent the mean ± S.D. ($n=4$ oocytes).

rent displayed inward rectification (Fig. 3B). Such a marked increase of Na^+ leakage currents after MTSET incubation

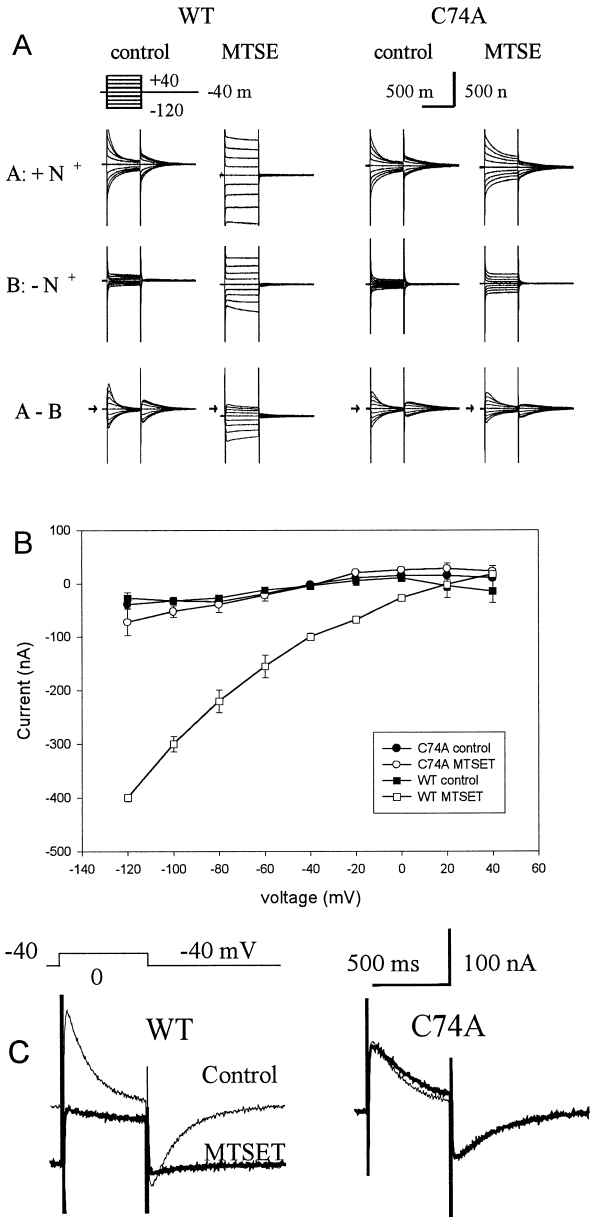

Fig. 3. Voltage-jump analysis for WT and C74A GAT1. A: The membrane potential was held at −40 mV and stepped to various test potentials between −140 and 40 mV in 10 mV increments (traces are shown at 20 mV increments for clarity). The top two rows (A and B) show raw traces associated with these jumps, recorded with and without Na^+, respectively. The third row (A-B) shows records obtained by subtraction within the column; this subtraction isolates the components dependent on Na^+. Oocytes expressing WT or C74A GAT1 were treated with MTSET (3.75 mM) for 30 min and then washed. This yielded the data in the second and fourth column. B: Current-voltage relationship of MTSET-activated Na^+-dependent leakage current, isolated as in panel A. The current was averaged over the time interval between 500 and 530 ms after the start of the test pulse. Data points represent the mean ± S.D. (n=2–3 oocytes). C: Subtracted voltage-jump relaxations, before and after MTSET incubation at 0 mV, have been superimposed for comparison of capacitive charge movements due to ion binding. Note also that WT GAT1 treated with MTSET gives a more negative current baseline relative to the control baseline, corresponding to the increased leakage current shown in Fig. 1.

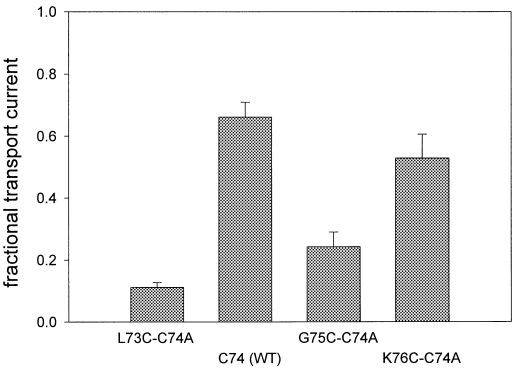

Fig. 4. Cysteine scanning mutagenesis at positions 73–76. Incubation was carried out in 1.0 mM MTSET for 5 min, and the effect on transport-associated current is shown. Bars represent the mean ± S.D. of triplicate samples.

was not observed in oocytes expressing the C74A mutant (Figs. 1 and 3B) or in uninjected oocytes (data not shown). These leakage currents were not blocked by addition of the GABA uptake inhibitor NO-05-711 (data not shown).

3.4. Cysteine scanning mutagenesis at positions 73–76

Three mutants were constructed, introducing a cysteine residue at each of the positions L73, G75, and K76. In each mutant, we also changed the residue at position 74 from the WT cysteine to alanine. The reactivity of these engineered cysteine residues to extracellular MTSET was assessed (Fig. 4). At positions 73 and 75, reactivity to MTSET was substantially greater than the WT (position 74) sensitivity, presumably due to enhanced accessibility. In addition, the 76C mutant showed a small increase in sensitivity to MTSET compared with WT. Thus, all four positions tested, 73 through 76, were indeed accessible to MTSET applied to the outside of the cell.

4. Discussion

The present study is the first to combine the use of substituted cysteine mutagenesis and electrophysiological measurements on neurotransmitter transporters. Each of four adjacent residues in the short HD1–2 loop was accessible to extracellularly applied, membrane-impermeant sulfhydryl reagents. Functional impairment of GAT1 due to treatment with membrane-impermeant MTSET or MTSES specifically involved the native cysteine at position 74, while membrane-permeant MTSEA appeared to react with multiple cysteine residues to abolish transporter function.

The results summarized above are consistent with the recent finding that the corresponding cysteine, C109, of the rat serotonin transporter (rSERT) is not necessary for normal function and reacts with externally applied MTSET and MTSES [8]. C109 of rSERT reacts rather slowly, as did C74 of GAT1 in our studies, requiring minutes of incubation at millimolar concentrations. We hypothesize that C74 is at least partially buried. Cysteine residues directly adjacent to C74 (positions 73 and 75) were substantially more reactive, presumably because the secondary structure of the HD1–2 loop positions these residues for greater exposure.

The HD1–2 stretch, which includes C74, may participate in the pore or translocation pathway of the transporter. Cova-

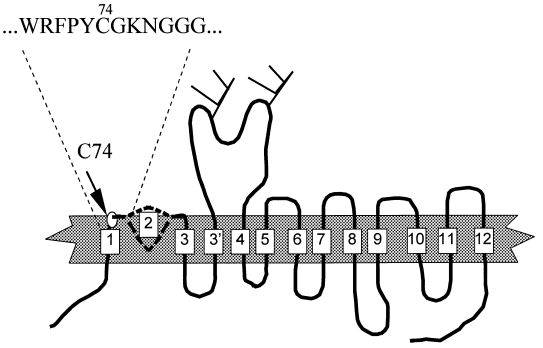

Fig. 5. Revised topological model for GAT1. The HD2 region is drawn here either as extracellular or as a reentrant membrane loop. The new hydrophobic domain 3′ proposed by both Bennett and Kanner [6] and Olivares et al. [7] is incorporated as well. Beginning with HD4, the topology remains identical to the original theoretical model [1]. Also shown is the partial amino acid sequence listing of the HD1–HD2 region, which includes W68, R69, and C74.

lent modification of C74 both abolished GABA transport-associated current and also altered the ion-transporter interaction: Na^+ binding was eliminated, as inferred from loss of charge movements, and Na^+ and Li^+ leakage currents were increased. In addition, non-Na^+/Li^+ leakage currents were induced, possibly due to K^+ and/or Cl^- permeation at the C74-modified transporter. Manipulations that decrease capacitive charge movements while increasing uncoupled leakage currents are thought to affect the permeation pathway of ion-coupled transporters [20,21]. NO-05-711 blocks previously described GAT1-associated currents [20]. Loss of NO-05-711 sensitivity, presumably because the inhibitor cannot bind to MTSET-treated GAT1, is also consistent with the altered Na^+ interaction [20]. We find that C74 modification eliminates Na^+ binding transient currents, and that inhibitor binding depends strongly on Na^+ binding by the transporter [20].

Nearby mutations in HD1 at tryptophan 68 (W68), a residue previously hypothesized to be involved in ion binding and/or permeation [20], altered MTSET accessibility of C74. Arginine 69, which directly neighbors W68, is also critical for the function of GAT1 [22]. Moreover, HD1, as well as the HD1-2 loop, including C74, is highly conserved throughout the neurotransmitter transporter superfamily as well as in putative Na^+-dependent transporters in the bacteria, *Haemophilus influenzae* [23] and *Methanococcus jannaschii* [24], suggesting that the domain forms a specialized structure critical for a particular function. Bennett and Kanner [6] and Olivares et al. [7] studied glycosylation in the HD2–3 loop, which is intracellular in the original model, and proposed that a new HD domain (HD3′ in Fig. 5) is formed by a stretch of amino acids previously thought to reside in the extracellular HD3–4 loop. Those authors also suggested that the HD1–2 loop is intracellular, because (1) the loop was not glycosylated, (2) HD1 did not translocate a fused reporter sequence in in vitro translation assays, and (3) MTSET did not block function despite the presence of C74 in HD1–2. We do not show 1.5 mM MTSET for 5 min produced $< 10\%$ block in the experiments of Bennett and Kanner [6], while 1 mM and 2 mM MTSET for 5 min were able to produce 35% to 45% (respectively) block of function in our experiments. Nonetheless our result, in addition to the observation that other nearby cys residues are even more sensitive to MTSET, leads to our conclusion this stretch of four residues in the HD1–2 loop is exposed to externally applied membrane-impermeant cysteine reagents.

Clark [9] found that the amino-end of the HD3–4 loop was accessible to protease applied on the cytoplasmic side of a chimeric GAT1 molecule with a prolactin tag fused downstream of HD5. In the same construct, the HD2–3 loop was not cleaved by the protease. These findings are consistent with the extracellular localization of the HD2–3 loop, inferred from glycosylation experiments, and with the presence of a new hydrophobic domain, HD3′.

Topological models for GAT1 must now accommodate the observations that sequences in both the HD1–2 loop (our data) and the HD2–3 loop [6,7,9] are extracellular. In one possible explanation, HD2 does not span the membrane; in another, HD2 forms a reentrant loop that dips into the membrane without completely traversing it (Fig. 5). Similar domains are described for K^+ channels [25], glutamate receptors [26,27], and aquaporin [28,29].

Acknowledgements: We thank Brad Henkle and Hai-Rong Li for help with the oocytes. This work was supported by grants from the National Institutes of Health (NS-11756, DA-09121).

References

[1] Guastella, J.G., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., Midel, M.C., Davidson, N., Lester, H.A. and Kanner, B. (1990) Science 249, 1303–1306.
[2] Kanner, B.I. and Schuldiner, S. (1987) CRC Crit. Rev. Biochem. 22, 1–38.
[3] Kavanaugh, M.P., Arriza, J.L., North, R.A. and Amara, S.G. (1992) J. Biol. Chem. 267, 22007–22009.
[4] Mager, S., Naeve, J., Quick, M., Guastella, J., Davidson, N. and Lester, H.A. (1993) Neuron 10, 177–188.
[5] Turk, E., Kerner, C.J., Lostao, M.P. and Wright, E.M. (1996) J. Biol. Chem. 271, 1925–1934.
[6] Bennett, E.R. and Kanner, B.I. (1997) J. Biol. Chem. 272, 1203–1210.
[7] Olivares, L., Aragon, C., Gimenez, C. and Zafra, F. (1997) J. Biol. Chem. 272, 1211–1217.
[8] Chen, J.G., Liu-Chen, S. and Rudnick, G. (1997) Biochemistry 36, 1479–1486.
[9] Clark, J.A. (1997) J. Biol. Chem. 272, 14695–14704.
[10] Akabas, M.H., Stauffer, D.A., Xu, M. and Karlin, A. (1992) Science 258, 307–310.
[11] Xu, M. and Akabas, M.H. (1993) J. Biol. Chem. 268, 21505–21508.
[12] Stauffer, D.A. and Karlin, A. (1994) Biochemistry 33, 6840–6849.
[13] Lu, Q. and Miller, C. (1995) Science 268, 304–307.
[14] Liu, Y., Jurman, M.E. and Yellen, G. (1996) Neuron 16, 859–867.
[15] Nowak, M.W., Kearney, P.C., Sampson, J.R., Saks, M.E., Labarca, C.G., Silverman, S.K., Zhong, W., Thorson, J., Abelson, J.N., Davidson, N., Schultz, P.G., Dougherty, D.A. and Lester, H.A. (1995) Science 268, 439–441.
[16] Quick, M.W. and Lester, H.A. (1994) in: T., Narahashi (Ed.), Ion Channels of Excitable Cells, Academic Press, San Diego, pp. 261–279.
[17] Wang, J.B., Moriwaki, A. and Uhl, G.R. (1995) J. Neurochem. 64, 1416–1419.
[18] Holmgren, M., Liu, Y., Xu, Y. and Yellen, G. (1996) Neuropharmacology 35, 797–804.
[19] Olami, Y., Rimon, A., Gerchman, Y., Rothman, A. and Padan, E. (1997) J. Biol. Chem. 272, 1761–1768.
[20] Mager, S., Kleinberger-Doron, N., Keshet, G.I., Davidson, N., Kanner, B.I. and Lester, H.A. (1996) J. Neurosci. 16, 5405–5414.
[21] Lester, H.A., Cao, Y. and Mager, S. (1996) Neuron 17, 807–810.
[22] Pantanowitz, S., Bandahan, A. and Kanner, B.I. (1993) J. Biol. Chem. 286, 3222–3225.
[23] Fleischmann, R.D., Adams, M.D., White, O., Clayton, R.A.,

Kirkness, E.F., Kerlavage, A.R., Bult, C.J., Tomb, J.F., Dougherty, B.A. and Merrick, J.M. (1995) Science 269, 496–512.
[24] Bult, C.J. et al. (1996) Science 273, 1058–1073.
[25] Miller, C. (1991) Science 252, 1092–1096.
[26] Hollmann, M., Maron, C. and Heinemann, S. (1994) Neuron 13, 1331–1343.
[27] Bennett, J.A. and Dingledine, R. (1995) Neuron 14, 373–384.
[28] Cheng, A., van Hoek, A.N., Yeager, M., Verkman, A.S. and Mitra, A.K. (1997) Nature 387, 627–630.
[29] Walz, T., Hirai, T., Murata, K., Heymann, J.B., Mitsuoka, K., Fujiyoshi, Y., Smith, B.L., Agre, P. and Engel, A. (1997) Nature 387, 624–627.