Structural Determinants for the Action of Grayanotoxin in D1 S4-S5 and D4 S4-S5 Intracellular Linkers of Sodium Channel Alpha Subunits

Hiroshi Maejima*, Eiji Kinoshita[†], Tsunetsugu Yuki[†], Masuhide Yakehiro[‡], Issei Seyama[†] and Kaoru Yamaoka[†1]

[†]*Department of Physiology, *Institute of Health Sciences, School of Medicine, Hiroshima University, Hiroshima, Kasumi 1-2-3, Hiroshima 734-8551, and* [‡]*Division of Physiology, Department of Clinical Engineering, Faculty of Health Sciences, Hiroshima International University, 555-36 Gakuendai, Kurose-cho, Kamo-gun, Hiroshima Prefecture, 732-0695, Japan*

[1]To whom correspondence should be addressed.

Mailing address: Kaoru Yamaoka

Department of Physiology, School of Medicine, Hiroshima University,

Kasumi 1-2-3, Hiroshima 734-8551, Japan

Tel: +81-82-257-5120

Fax: +81-82-257-5124

e-mail: kyamaok@hiroshima-u.ac.jp

Abbreviations used:

D, domain; GTX, grayanotoxin; HEK, human embryonic kidney; I_{Na}, Na^+ current; I-V, current-voltage; μ1, α-subunit of rat skeletal muscle Na^+ channel; rH1, α-subunit of rat heart Na^+ channel; S, segment; Each mutant channel will be referenced by the original amino acid followed by its number and introduced amino acid.

ABSTRACT

We located a novel binding site for grayanotoxin on the cytoplasmic linkers of voltage-dependent cardiac (rH1) or skeletal-muscle (μ1) Na^+ channel isoforms (segments S4-S5 in domains D1 and D4), using the alanine scanning substitution method. GTX-modification of Na^+ channels, transiently expressed in HEK 293 cells, was evaluated under whole-cell voltage clamp, from the ratio of maximum chord conductance for modified and unmodified Na^+ channels. In μl, mutations K237A, L243A, S246A, K248A, K249A, L250A, S251A or T1463A, caused a moderate, but statistically significant decrease in this ratio. On making corresponding mutations in rH1, only L244A dramatically reduced the ratio. Because in μ1, the serine at position 251 is the only heterologous residue with respect to rH1 (Ala-252), we made a double mutant L243A&S251A to match the sequence of μ1 and rH1 in S4-S5 linkers of both domains. This double mutation resulted in a significant decrease in the ratio, to the same extent as L244A substitution in rH1 did, indicating that the site at Leu-244 in rH1 or at Leu-243 in μ1 is a novel one, exhibiting a synergistic effect of grayanotoxin.

***Keywords*:** grayanotoxin, sodium channel, S4-S5 linker, whole-cell patch clamp, HEK 293

INTRODUCTION

Grayanotoxin belongs to the class of so-called lipid-soluble toxins which include batrachotoxin (BTX), veratridine, and aconitine. The unique actions of these toxins on voltage-dependent Na^+ channels consist of a hyperpolarizing shift in voltage-dependency of channel activation and elimination of fast Na-inactivation. While all toxins in this class are potentially useful for elucidating the molecular basis of Na^+ channel gating, GTX has the distinct advantage that the relevant interaction sites on the GTX molecule are already known, as a result of exhaustive analyses of its structure-activity relationship (1-3). The methyl group on residue 10 of the molecule's β-surface and the hydroxyl groups on several residues of its β [3, 5, 6]- and α-surface [15, 16] are essential to the pharmacologic action of GTX. Moreover, GTX action has a unique dependency on the state of the Na^+ channel: GTX binds only to the open state and dissociates from the closed state (4).

Previously, we reported that transmembrane segments D1S6 and D4S6 are required for GTX-binding to the Na^+ channel (5, 6). Within these segments, a number of putative binding sites on the μ1 Na^+ channel isoform (Ile-433, Asn-434, Leu-437, Ile-1575, Phe-1579 and Tyr-1586) may interact with the α-surface of the GTX molecule, whose pharmacologic action is effected within a hydrophobic micro-environment (3). Tyr-1586 in D4S6 has been found to provide a unique binding site for GTX, to the exclusion of the other known lipid-soluble toxins (6). Moreover, we have attributed the difference in GTX sensitivity of the Na^+ channel isoforms, μ1 and rH1, to a critical residue (Ser-251) in the intracellular loop of D1S4-S5 (7). Kimura et al. (7) deduced particular GTX binding sites by making several chimeras targeting the heterologous amino acid residues of μ1 and rH1, and then noting which chimeras showed reduced sensitivity to GTX. Using such a strategy, we may have overlooked critical sites on these Na^+ channel isoforms in conserved regions of their amino acid sequence, which nonetheless have intimate involvement in GTX-action. To overcome

this shortcoming, we thought it worthwhile to examine the S4-S5 linkers in D1 and D4 with the alanine scanning substitution method to see whether novel amino acid residues (in addition to those already reported) are critical for GTX-action.

MATERIALS AND METHODS

Construction of Alanine Scanning Mutants of Na^+ Channels

Alanine scanning mutants of Na^+ channels were constructed using two cDNA clones encoding the rat skeletal muscle, and cardiac α-subunits, μ1 (8) and rH1 (9). To construct the mutants by alanine substitution, we followed our method described previously (5-7, 10). All of the resulting mutants were confirmed with restriction mapping and sequencing using an ABI PRISM^{TM} 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Transient Transfection and Cell Culture

The point-mutated cDNA clones were inserted into a mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), and were then transiently co-transfected with CD8 cDNA into HEK 293 cells using the SuperFect transfection reagent (Qiagen, Hilden, Germany). The cells were grown to 50% confluence in DMEM (Invitrogen), containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA), 30 units/ml penicillin G (Invitrogen) and 30 μg/ml streptomycin (Invitrogen), in 60-mm tissue culture dishes, and in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C. The transfected cells were used for electrophysiological experiments as late as 3 to 4 days after transfection. Transfection-positive cells were identified by immunobeads (CD8-Dynabeads, Dynal, Oslo, Norway) before Na^+ current (I_{Na}) recording.

Electrophysiological Recording

Macroscopic I_{Na} from the transfected cells was measured using the whole-cell variation of the patch clamp method. The bath solution contained (in mM): 70 NaCl, 67 *N*-metyl-*D*-glucamine, 1 $CaCl_2$, 1.5 $MgCl_2$, 10 glucose, and 5 HEPES (pH 7.4). The pipette

solution contained (in mM): 70 CsF, 60 CsCl, 12 NaF, 5 ethylene-bis (oxonitrilo) tetraacetic acid and 5 HEPES (pH 7.2). To assess the effects of GTX on whole-cell I_{Na}, 300 μM of GTX I was added to the pipette solution, because GTX is known to act intracellularly (11). Data are presented as mean ± SD (number of observations), unless otherwise indicated.

RESULTS

Effect of GTX on wild type μ1 and rH1

Because GTX exclusively binds to Na^+ channels in their open state (4-7, 12), repetitive depolarizing pulses were applied to induce GTX modification. Following these repetitive conditioning pulses, test pulses to various membrane potentials induced a characteristic sustained current (Figs. 1A2 and B2). Without repetitive prepulses, the majority of channels opened and inactivated normally, although a slight increase in non-inactivating I_{Na} at the end of test pulses indicated that a small fraction of the channels were modified during the test pulse itself (Figs. 1A1 and B1).

Since each transfected cell expresses the Na^+ channel at different densities, we used the maximum conductance to standardize data from different cells as described previously (6, 7, 12). The I-V relationships for unmodified I_{Na} through wild type rH1 channels and its mutant forms (rH1-L244A) are given in Figs. 1A3 and B3. The continuous line was fitted to peak I_{Na} at membrane potentials from 0 to +60 mV and the chord conductance was estimated from the slope. However, once 100 conditioning pulses were applied, an entirely different family of sustained currents through modified sodium channels was obtained (Figs. 1A2 and B2). For GTX-modified channels, I_{Na} at the end of a 160 msec test pulse was plotted against the membrane potential, and the chord conductance was estimated from the slope of the I-V relationship between -50 and +50 mV (dashed line in Figs. 1A3 and B3). Common pharmacological characteristics of various GTX analogues are summarized as follows: 1) single channel conductance of modified channels (wild-type isoforms) decreases to one-third; 2) the open channel probabilities are in the range from 0.78 to 0.8; and consequently 3) any difference in GTX action among Na^+ channel isoforms should be due to a change in the number of channels modified (7, 12). Therefore, it is reasonable to use the ratio of chord conductances of GTX-modified to unmodified channels as a measure of channel sensitivity to

GTX modification, which is referred to as the relative chord conductance.

Comparison of kinetic parameters of channel gating among mutant channels

To assess the kinetic properties of mutant and wild-type Na^+ channels, the time to peak I_{Na} was measured as estimate of activation kinetics and the time constant for the falling phase (τ_f) of I_{Na} as that of the inactivation process. As shown in Fig. 2, all measured values for the mutants of μ1 and rH1 were similar each other, indicating that the introduced mutations did not significantly modify the kinetics of these Na^+ channels.

Alanine substitutions within intracellular loops of D1S4-S5 and D4S4-S5 in μ1

We systematically substituted each amino acid in D1S4-S5 for alanine, one residue at a time, between Lys-237 and Asp-252. The same alanine scanning substitution method was also applied from Thr-1463 to Asn-1477 in D4S4-S5. In D1, the relative chord conductance for K237A, L243A, S246A, K248A, K249A, L250A and S251A was noticeably deceased by 37%, from 0.35 to around 0.22 (Fig. 3A). On substitution of amino acids in D4, a significant decrease in the relative chord conductance, comparable to the effect produced by D1 substitutions, was observed only in the case of T1463A (Fig. 3B). HEK 293 cells transfected with four mutant constructs of μ1, V247A, P1473A, L1475A and N1477A, did not express sufficient numbers of Na^+ channels to permit accurate measurements of I_{Na}.

Systematic substitution of amino acids in S4-S5 intracellular loops of D1 and D4 in rH1 with alanine

Only amino acids in the S4-S5 linkers of μ1 that significantly modified GTX-action were examined in rH1. Mutants of K238A, S247A, K249A, K250A and T1647A did not affect the GTX action. Only L244A dramatically decreased the relative chord conductance

by some 80%, from 0.22 to 0.04 (Fig. 3C). However, mutation of the homologous residue in μ1, L243A, resulted in only a moderate decrease in relative conductance of 34%, from 0.35 to 0.23. Since there is only one non-homologous amino acid in S4-S5 of D1 and D4 in each Na^+ channel (Fig. 3D; Ser-251 in μ1 and Ala-252 in rH1), we hypothesized that this amino acid difference may strengthen GTX-action in the μ1 isoform. Accordingly, a double mutant, μ1-L243A&S251A, was constructed to match the amino acid sequence of μ1 and rH1 in the indicated segments. This double mutant of μ1 actually exhibited a substantial decrease in relative chord conductance from 0.35 to 0.10, which was equivalent to the L244A mutant in rH1 (Fig. 3A; filled column).

DISCUSSION

In the previous paper by Kimura et al. (7), we attempted to identify the site responsible for GTX-action by making chimeras with different combinations of Na^+ channel cDNAs encoding μ1 and rH1. The fundamental assumption was that the difference in the response of Na^+ channel isoforms to GTX comes from non-homologous amino acid residues in μl and rH1. Using this strategy, Kimura et al. (7) were able to deduce that residue 251 in μ1 is a critical site responsible for the difference in GTX-sensitivity between μ1 and rH1. In the present study, the alanine scanning method shed new light on additional sites in the same S4-S5 linker in μ1 domain D1 by showing a moderate decrease in channel modification induced by GTX. However, corresponding mutations in rH1 did not lead to any such decrease with the exception of L244A. This remarkable mutation decreased channel modification much more than any of the mutations in the μ1 isoform. When we examine the entire sequence of the S4-S5 linker in both D1 and D4, we recognize that there is a difference of one amino acid residue, Ser-251 in μ1 and Ala-252 in rH1 (Fig. 3D). Thus, it is reasonable to suppose that the reduced GTX effect in rH1 may be due to the presence of Ala-252 instead of Ser at the corresponding site in μ1. The double mutant, L243A&S251A, did give a substantial decrease in GTX-induced channel modification (Fig. 3A in the last filled column). From these observations, it is concluded that Leu-244 in rH1 or at residue 243 in μ1 is critical to GTX-action. A similar finding was obtained by surveying mutants of insect para-Na^+ channels resistant to pyrethroids using mortality as a rough index of channel modification (13). These investigators reported that mutation of I287N in the D1S4-S5 linker confers a modest level of resistance to pyrethroids; moreover, insects having mutations in S6 segments plus the S4-S5 linkers in D1, D2 and D3 responded to the pyrethroid by a factor of one-hundred times less than wild type. In this connection, it is worthwhile to note certain

similarities in the pharmacological effects of pyrethroids and GTX on Na^+ channels: 1) both pyrethroids and GTX are known to prolong open time of Na^+ channels and to shift the sodium conductance-voltage curve in the hyperpolarizing direction (12, 14); 2) treatment with these toxins induced a characteristic, long-lasting opening of single Na^+ channels when rectangular pulses were applied (12, 14). However, some differences also exist: 1) GTX modifies Na^+ channels strictly in their open state (4), whereas pyrethroids can modify Na^+ channels in all states (14), including the channel's open configuration, which still has higher susceptibility to GTX than the resting or closed states; 2) single channel conductance of Na^+ channels modified by pyrethroids remains the same as that in control (14), whereas GTX modification reduces channel conductance to one-third of the control value (7, 12). Thus, S4-S5 linkers take part in the modification of channel gating whether the toxins used are GTX analogs or pyrethroids. Furthermore, there are some examples in which the S4-S5 linker is involved in the voltage dependence of Na^+ channel gating, although the domains involved can vary. Interactions between the inactivation gate (corresponding to the intracellular loop connecting D3 and D4) and the S4-S5 linkers of D3 and D4 have been suggested on the basis of site-directed mutagenesis (15-17). Examples of the involvement of S4-S5 linkers in *Paramyotonia congenita* are the I693T mutation in the S4-S5 linker of D2, which induced a hyperpolarizing shift of about 9 mV in the voltage dependence of activation without greatly affecting the inactivation process (18), and mutations in the S4-S5 linker in D4, which slowed fast inactivation and revealed that this S4-S5 linker may play an important role in mediating the coupling between activation and inactivation (19, 20). Additionally, the LQT 3 mutation interfered with inactivation by destabilizing the structure of the acceptor site for the inactivation particle (21), which is located in the S4-S5 linker of D3.

Possible involvement of the S4 segment in sensing the change in membrane potential has been argued, since the late Professor Numa's group determined the Na^+ channel cDNA

sequence (22). Using the cystein-substitution method, Yang and Horn (23) showed that during the operation of Na^+ channel gates, depolarization causes all four S4 segments to move outward to their active position. With the aid of site-directed fluorescent labeling, Cha et al. (24) were able to trace the functional role of the S4 segments of D1 and D2 to the nonimmobilizable component of gating charge (associated with channel inactivation) and that of the S4 segments of D3 and D4, to the immobilizable fraction. Taking into consideration the available information on the structure-function relationships of the Na^+ channel, we are tempted to speculate that the S4-S5 linker in D1 may take part in transferring the molecular information about GTX-binding to the voltage sensor on the S4 segment, thereby causing a shift in the voltage dependence of activation in a manner very similar to the pyrethroids.

ACKNOWLEDGMENTS

We would like to express our sincere gratitude to Dr. Keiji Imoto (National Institute for Physiological Sciences, Okazaki, Japan) for providing HEK 293 cells, and to Dr. Stephen M. Vogel (Department of Pharmacology, University of Illinois at Chicago, College of Medicine) for his critical reading of the manuscript. We wish to thank the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University for the use of their facilities. This work was supported by grants from the Ministry of Education and Culture of Japan to K.Y. (11470011) and E.K. (11770023, 14770014), and by a research grant for cardiovascular diseases from the Ministry of Health and Welfare to I.S. (11C-1).

REFERENCES

1. Masutani, T., Seyama, I., Narahashi, T. and Iwasa, J. (1981) *J. Pharmacol. Exp. Therap.* **217,** 812-819.
2. Tsuji, K., Kawanishi, T., Handa, S., Kamano, H., Iwasa, J. and Seyama, I. (1991) *J. Pharmacol. Exp. Therap.* **257,** 788-794.
3. Yakehiro, M., Yamamoto, S., Baba, N., Nakajima, S., Iwasa, J. and Seyama, I. (1993) *J. Pharmacol. Exp. Therap.* **265,** 1328-1332.
4. Yuki, T., Yamaoka, K., Yakehiro, M. and Seyama, I. (2001) *J. Physiol. (Lond)* **534,** 777-790.
5. Ishii, H., Kinoshita, E., Kimura, T., Yakehiro, M., Yamaoka, K., Imoto, K., Mori, Y. and Seyama, I. (1999) *Jpn. J. Physiol.* **49,** 457-461.
6. Kimura, T., Kinoshita, E., Yamaoka, K., Yuki, T., Yakehiro, M. and Seyama, I. (2000) *FEBS Lett.* **465,** 18-22.
7. Kimura, T., Yamaoka, K., Kinoshita, E., Maejima, H., Yuki, T., Yakehiro, M. and Seyama, I. (2001) *Mol. Pharmacol.* **60,** 865-872.
8. Trimmer, J. S., Cooperman, S. S., Tomiko, S. A., Zhou, J., Crean, S. M., Boyle, M. B., Kallen, R. G., Sheng, Z., Barchi, R. L., Sigworth, F. J., Goodman, R. H., Agnew, W. S. and Mandel, G. (1989) *Neuron* **3,** 33-49.
9. Rogart, R. B., Cribbs, L. L., Muglia, L.K., Kephart, D. and Kaiser, M. W. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8170-8174.
10. Kinoshita, E., Maejima, H., Yamaoka, K., Konno, K., Kawai, N., Shimizu, E., Yokote, S., Nakayama, H. and Seyama, I. (2001) *Mol. Pharmacol.* **59,** 1457-1463.
11. Seyama, I., Yamada, K., Kato, R., Masutani, T. and Hamada, M. (1988) *Biophys. J.* **53,** 271-274.

12. Yakehiro, M., Yuki, T., Yamaoka, K., Furue, T., Mori, Y., Imoto, K. and Seyama, I. (2000) *Mol. Pharmacol.* **58,** 692-700.

13. Pittendrigh, B., Reenan, R., ffrench-Constant, R.H. and Ganetzky, B. (1997) *Mol. Gen. Genet.* **256,** 602-610.

14. Narahashi, T. (1996) *Pharmacol. Toxicol.* **79,** 1-14.

15. Tang, L., Kallen, R.G. and Horn, R. (1996) *J. Gen. Physiol.* **108,** 89-104.

16. Smith, M. R. and Goldin, A. L. (1997) *Biophys. J.* **73,** 1885-1895.

17. McPhee, J. C., Ragsdale, D. S., Scheuer, T. and Catterall W. A. (1998) *J. Biol. Chem.* **273,** 1121-1129.

18. Plassart-Schiess, E., Lhuillier, L., George, A. L. Jr., Fontaine, B. and Tabti, N. (1998) *J. Physiol. (Lond)* **507,** 721-727.

19. Fleischhauer, R., Mitrovic, N., Deymeer, F., Lehmann-Horn, F. and Lerche, H. (1998) *Pflügers Arch.* **436,** 757-765.

20. Alekov, A. K., Peter, W., Mitrovic, N., Lehmann-Horn, F. and Lerche, H. (2001) *Neurosci. Lett.* **306,** 173-176.

21. Dumaine, R. and Kirsch, G. E. (1998) *Am. J. Physiol.* **274,** H477-487.

22. Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayano, T., Ikeda, T., Takahashi, H., Nakayama, H., Kanaoka, Y., Minamino, N., Kangawa, K., Matsuo, H., Raftery, M. A., Hirose, T., Inayama, S., Hayashida, H., Miyata, T. and Numa, S. (1984) *Nature* **312,** 121-127.

23. Yang, N. and Horn, R. (1995) *Neuron* **15,** 213-218.

24. Cha, A., Ruben, P. C., George, A. L. Jr., Fujimoto, E. and Bezanilla, F. (1999) *Neuron* **22,** 73-87.

FIGURE LEGENDS

FIG. 1. GTX modification of Na^+ channels. Differences in potency of GTX I between wild-type rH1 and mutant L244A. I_{Na} families and I-V relations for unmodified peak I_{Na} and for GTX I-modified steady-state I_{Na} in wild type rH1 (A) and its mutant L244A (B). Even in the presence of 300 μ M GTX, the currents were scarcely modified without preconditioning pulses as shown in A1 and B1. Thus, control currents (unmodified currents) were taken in the presence of intracellular GTX. Modified current (open circles in I-V plots of A3 and B3) was induced by a train of 100 conditioning prepulses (pulse potential, –20 mV; pulse duration and interpulse interval, 6 msec and 100 ms; holding potential, –120 mV), and assayed with a 160-msec test pulse to variable potentials between –140 mV and +50 mV, incremented in 10-mV steps (A2 and B2). Predominantly unmodified current (filled squares in I-V plots of A3 and B3) was obtained without application of conditioning prepulses and measured by peak I_{Na} (A1 and B1).

FIG. 2. Similar inactivation and activation kinetics of wild type and mutant channels. A1-C1: Relationship between time-to-peak I_{Na} and membrane potential in HEK cells expressing mutations in the S4-S5 linker of μl domain D1 (A1), the S4-S5 linker of μl domain D4 (B1), and the S4-S5 linker of rH1 domains D1 and D4 (C1). A2-C2: Relationship between the time constant (τ_f) for the falling phase of I_{Na} and membrane potential in the above-described mutants of μ1 D1 (A2), μ1 D4 (B2), and rH1 D1 plus D4 (C2). For the sake of clarity, mean values without error bars are given for the indicated mutant channels. Number of observations was 6 each for wild type μ1 and rH1 isoforms, and 4 for mutant channels.

FIG. 3. Effect of GTX I on wild type and mutant channels. (A) Mutants of S4-S5 linker in μl D1. (B) Mutants in D4 of μ1. (C) Mutants in both rH1 D1 and D4. Hatched bars in *A* and *B* indicate that moderate suppression of GTX-action is recognizable; filled bars in *A* and *C* show that the observed suppression is sizeable. (D) Summary of the sites of action of GTX in μ1. Squares with bold line indicate marked suppression of GTX-action commonly observed in both μ1 and rH1; squares with thinner line show the moderate suppression observed only in μ1. For the sake of convenience, amino acid sequences for the S4-S5 linkers in rH1 domains D1 and D4 are also displayed (amino acid sequence numbered from N terminus), and the sites of action for GTX in D1S6 and D4S6, as reported in Ref. 5 and 6, are included.

Fig. 1 Maejima *et al.*

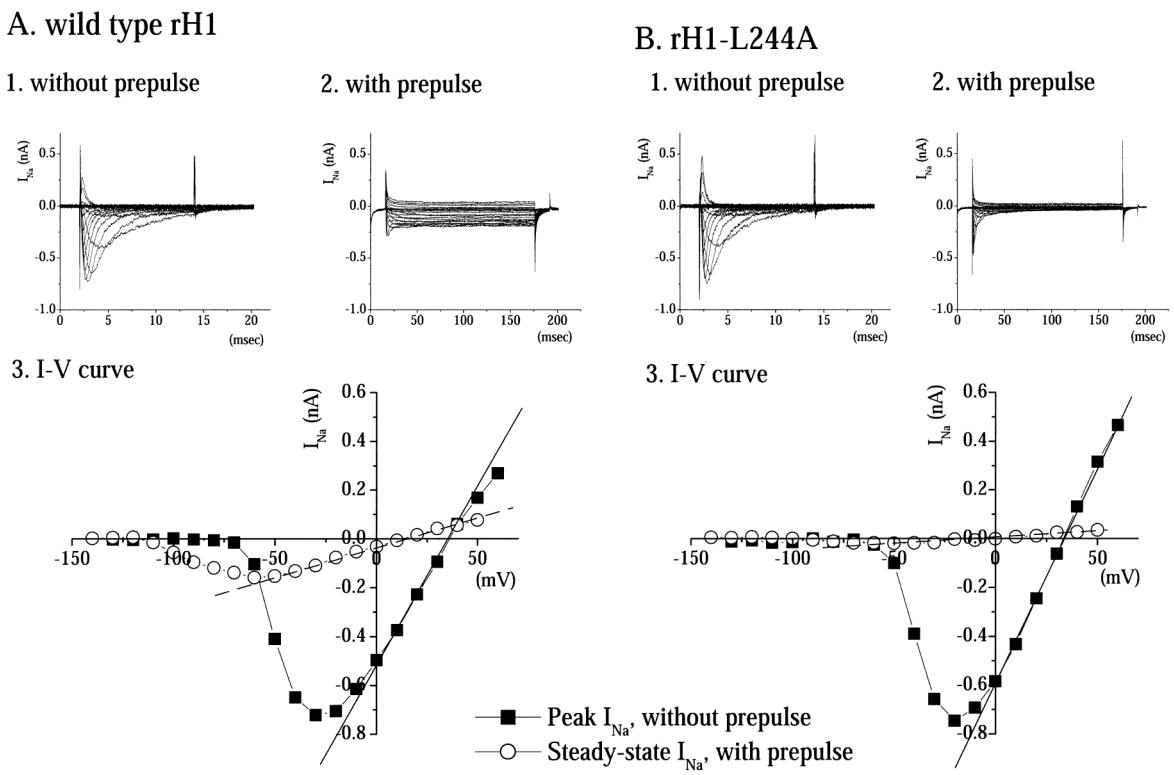

Fig. 2 Maejima *et al.*

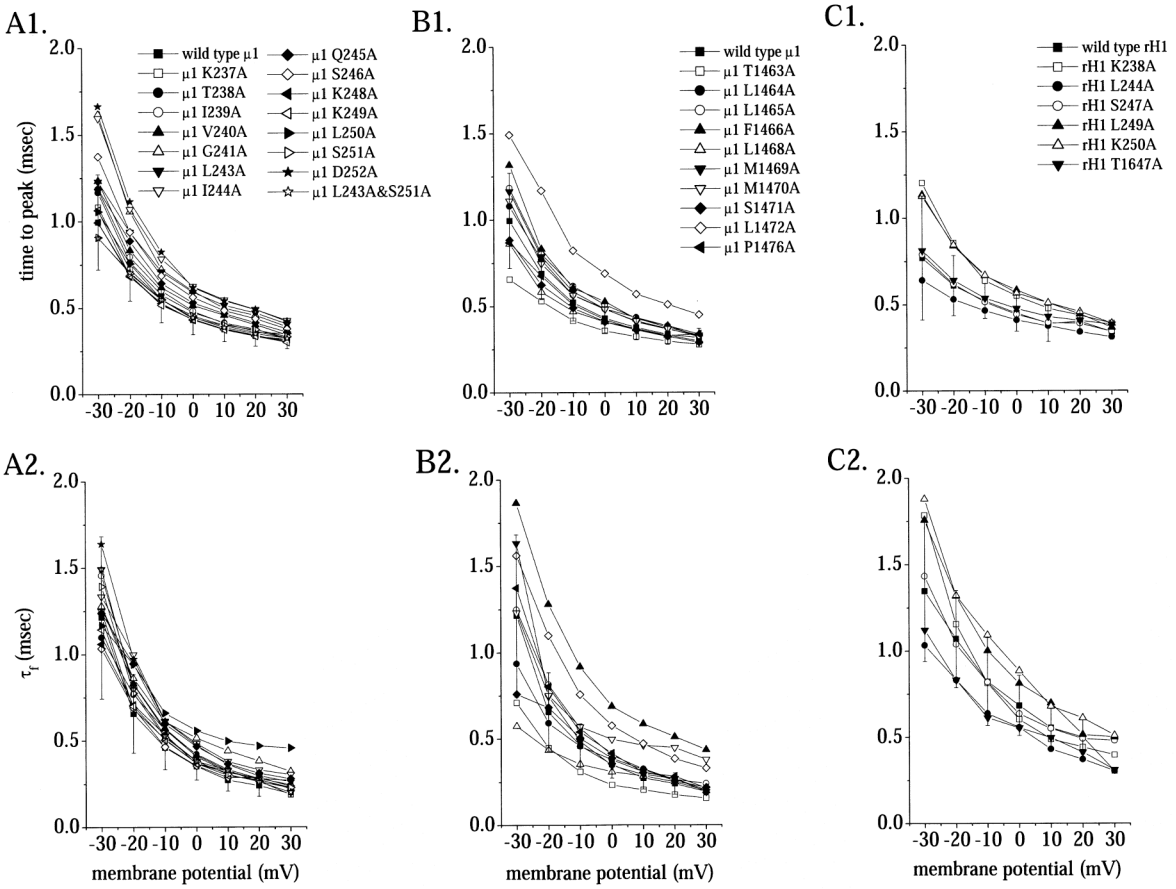

Fig. 3 Maejima *et al.*