The Acid-sensitive Ionic Channel Subunit ASIC and the Mammalian Degenerin MDEG Form a Heteromultimeric H⁺-gated Na⁺ Channel with Novel Properties*

(Received for publication, August 4, 1997, and in revised form, September 3, 1997)

Frederic Bassilana, Guy Champigny, Rainer Waldmann, Jan R. de Weille, Catherine Heurteaux, and Michel Lazdunski

From the Institut de Pharmacologie Moleculaire et Cellulaire, CNRS-UPR 411, 660, route des Lucioles, Sophia Antipolis, 06560 Valbonne, France

Proton-gated channel cations are acid sensors that are present in both sensory neurons and in neurons of the central nervous system. One of these acid-sensing ion channels (ASIC) has been recently cloned. This paper shows that ASIC and the mammalian degenerin MDEG, which are colocalized in the same brain regions, can directly associate with each other. Immunoprecipitation of MDEG causes coprecipitation of ASIC. Moreover, coexpression of ASIC and MDEG subunits in Xenopus oocytes generates an amiloride-sensitive H⁺-gated Na⁺ channel with novel properties (different kinetics, ionic selectivity, and pH sensitivity). In addition, coexpression of MDEG with mutants of the ASIC subunit can create constitutively active channels that become completely nonselective for Na⁺ versus K⁺ and H⁺-gated channels that have a drastically altered pH sensitivity compared with MDEG. These data clearly show that ASIC and MDEG can form heteromultimeric assemblies with novel properties. Heteromultimeric assembly is probably used for creating a diversity of H⁺-gated cation channels acting as neuronal acid sensors in different pH ranges.

H⁺-gated cation channels are ligand-gated ion channels activated by the simplest possible ligand, the proton. In nociceptive neurons these channels are thought to be responsible for the sensation of pain that accompanies tissue acidosis (1–3), particularly during inflammation and ischemic conditions. H⁺-gated cation channels are also present in neurons of the central nervous system (2), where their physiological role remains to be established. We have recently cloned a proton-gated cation channel (ASIC, acid sensing ion channel 1) (4). The closest structural homologue of ASIC is MDEG (5, 6). MDEG is a mammalian degenerin. Upon the same mutations that in Caenorhabditis elegans degenerins induce degeneration of specific neurons (7,8), MDEG can also acquire constitutive Na⁺ channel activity that becomes toxic for the cells in which it is expressed (5). In addition, native MDEG also behaves as a H⁺-gated Na⁺ channel.

The variety of H⁺-gated cation channels with different ion selectivities, pH dependencies, and kinetics described in sensory neurons (1,9,10), as well as in neurons of the central nervous system (2), suggests that ASIC and MDEG are probably only the first two members of this novel ion channel family and that other genes for new H⁺-activated Na⁺ channels remain to be discovered. However, the existence of different genes is probably only one of the ways used to create a diversity of H⁺-gated cation channels. Many other types of ion channels are known to form heteromultimers of structurally related subunits, resulting in the assembly of channels with novel properties (11). Furthermore, other structural homologies of the ASIC and MDEG channels, such as the epithelial amiloride-sensitive Na⁺ channel (12–18) and the degenerins of the nematode C. elegans, which are believed to be mechanosensitive channels (19–21), require heteromultimeric subunit assembly for their function. All these considerations taken together suggest that new channels might be formed by heteromultimers of ASIC and MDEG subunits. This paper provides both electrophysiological and biochemical evidence that ASIC, together with MDEG, form heteromultimeric channels with properties distinct from those of the parental subunits.

EXPERIMENTAL PROCEDURES

In Situ Hybridization—Experiments were performed on adult Wistar rats by using standard procedures (4). Brains were fixed in ice-cold 4% (w/v) paraformaldehyde, 0.1 M sodium phosphate buffer solution (PBS, pH 7.4) for 8 h and then immersed overnight at 4 °C in a 20% sucrose/PBS solution. Frozen sections (10 µm) were cut on a cryostat (Leica) at −25 °C, collected on 3-aminopropylmethysilane-coated slides and stored at −20 °C until use. Four antisense oligonucleotides, complementary to the rat cDNA sequences of ASIC and MDEG, were used to detect ASIC and MDEG transcripts, respectively. The sequences of the oligonucleotides were: ASIC, 5′-GGTGTTAGAAGAGATCTCAGCGTCAATGCTCT-3′ or 5′-AACAGACGACCATCTGGTATTATCTGAGA-3′ and MDEG, 5′-ATGGACACGACTCTGGACACATGGCCAAACAC-3′ or 5′-GTCTACATGTTCGGTGGACGTCGCTG-3′.

Probes were 3′-end-labeled with [α-33P]dATP (3000 Ci/mmol, ICN Radiochemicals) by terminal deoxynucleotidyltransferase. Sections were treated consecutively with 0.1 M glycine in PBS for 10 min, PBS for 3 min, 5 µg/ml proteinase K diluted in 0.1 M Tris, 50 mM EDTA (pH 8.0) for 15 min at 37 °C, 4% paraformaldehyde/PBS (pH 7.2) for 5 min. Slides were then rinsed 10 min in PBS, acetylated for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine, and dehydrated. Hybridization was carried out overnight at 37 °C in hybridization buffer (50% deionized formamide, 10% dextran sulfate, 500 µg/ml denatured salmon sperm DNA, 1% Denhardt's, 5% Sarcosyl, 250 µg/ml yeast tRNA, 20 mM dithiothreitol, 20 mM NaPO₄ in 2 × SSC, and the radiolabeled probe (0.2 ng/ml, 8 × 10⁶ dpm/µg). After hybridization, slides were washed in 1 × SSC at room temperature for 30 min before dehydration, drying, and exposure to Hyperfilm-βmax (Amersham Corp.) for 6 days. Slides were then dipped in Ilford K5 nuclear emulsion (diluted 1:1 with water) and exposed 4 weeks. Sections were then stained with cresyl violet and coverslipped. The specificity of labeling was verified by cold displacement of the radioactive probe with a 500-fold excess of unlabelled oligonucleotide and by the use of two specific oligonucleotide probes for each subunit complementary to conserved regions in either the coding or the noncoding sequences of the cDNAs.

*This work was supported by the Centre National de la Recherche Scientifique (CNRS), the Association de Recherche contre le Cancer (ARC), and the Association française contre les Myopathies (AFM). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† These authors contributed equally to this work.

§ To whom correspondence and reprint requests should be addressed: Institut de Pharmacologie Moleculaire et Cellulaire, CNRS, 660 route des Lucioles, Sophia Antipolis, Valbonne, France. Tel.: 33-4-93-95-77-02 or -03; Fax: 33-4-93-95-77-04; E-mail: ipmc@unice.fr or rainer@unice.fr.

1 The abbreviations used are: PBS, phosphate-buffered saline; BSA, bovine serum albumin.
ASIC and MDEG Form a Heteromultimeric H\(^+\)-gated Cation Channel

**Production of Recombinant Baculoviruses—**Primers were designed to add the FlagM2 epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) (Eastman Kodak Co.) to the NH\(_2\) terminus of MDEG and the T7tag epitope (Met-Ala-Ser-Thr-Gly-Gly-Gln-Gln-Met-Gly) (Novagen) to the NH\(_2\) terminus of ASIC. The tagged ASIC and MDEG codon sequences were amplified by polymerase chain reaction and subcloned in the pVL 1393 vector (PharMingen). SF9 cells were transformed with either pVL-ASIC-T7tag or pVL-MDEG-Flag vector, and recombinant baculovirus was isolated according to the “baculoGold” protocol (PharMingen).

**Expression of ASIC T7tag or MDEG-Flag Fusion Proteins and Immunoprecipitation—**7 \(\times\) 10\(^5\) SF9 cells were infected with 10\(^5\) recombinant baculovirus particles (ASIC-T7tag, MDEG-Flag, or both). Four days later, cells were harvested, washed in PBS, sonicated at 4 °C in solubilization buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM Triton X-100, 0, 1% desoxycholate, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM iodoacetamide) and subsequently centrifuged for 30 min at 80,000 \(\times\) g. An aliquot of the supernatant containing 50 \(\mu\)g of protein was incubated overnight with 10 \(\mu\)l of M2 anti-Flag mouse monoclonal antibody immobilized on Sepharose (Eastman Kodak Co.). After a 30 s 8000 \(\times\) g spin, the supernatant was saved and the pellet was washed six times in 500 \(\mu\)l of solubilization buffer and twice with PBS. Both supernatant and pellet were resolved by SDS-polyacrylamide gel electrophoresis (9% acrylamide) and transferred onto nitrocellulose membranes (Hybond C extra Amer sham). The blots were saturated 30 min with TBST (10 mM Tris-Cl pH 7.5, 50 mM NaCl, 0.1% Tween 20) containing 4% BSA, then incubated overnight at 4 °C with either M2 anti-Flag antibody (anti-MDEG-Flag) or anti-T7tag monoclonal antibody (anti-ASIC-T7tag) in TBST containing 2% BSA. After 5 washes with TBST, the blots were incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Jackson Immunotech) 1:10,000 in TBST containing 2% BSA for 1 h at room temperature, washed with TBST and revealed using a substrate for enhanced chemiluminescence detection (ECL, Pierce).

**Electrophysiology—**Oocytes were injected with 0.1 or 0.25 ng of cRNA and microelectrode voltage-clamp and patch-clamp recordings were performed two days after injection. For outside-out patch the pipettes contained: 140 mM KCl, 2 mM MgCl\(_2\), 5 mM EGTA, 10 mM Hepes (pH 7.4) (with KOH). The bath medium contained: 140 mM NaCl or (140 mM LiCl or 140 mM N-methyl-D-glucamine chloride), 2 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 10 mM Hepes (pH 7.4) (adjusted with HCl, NaOH, LiOH or tetramethyl ammonium hydroxide). Rapid pH changes from the initial pH of 7.4 were produced by rapid perfusion with bath solution adjusted to the pH values indicated in Figs. 2–4. Data were sampled at 2 kHz and filtered at 500 Hz for analysis (Biopatch software).

**RESULTS AND DISCUSSION**

The in situ experiments performed on whole brain sections shows a widely overlapping expression of ASIC and MDEG mRNAs (Fig. 1A). The highest expression levels were detected in the olfactory bulb, neo and allocortical regions, dentate granule cells, as well as in pyramidal cells of CA1–CA3 subfields of the hippocampal formation, habenula, basolateral amygdaloid nuclei, and in the Purkinje and granule cells of the cerebellum. A diffuse expression was observed over most other regions of the basa ganglia, including thalamic nuclei, substantia nigra, striatum and globus pallidus, hypothalamus, midbrain, pons, and medulla. ASIC and MDEG transcripts were expressed at low levels in choroid plexus. Coexpression of both transcripts in the same type of neurons was observed in all regions of gray matter, which are strongly labeled. Fig. 1A, panels c–d, show an example of the high degree of colocalization of ASIC and MDEG in the Purkinje and granule cells of the cerebellar cortex. These results are consistent with observations made by others (22). This colocalization led us to analyze whether MDEG and ASIC could interact to form a proton-activated channel and whether this new channel had different properties from those of the parental subunits expressed alone.

The direct demonstration of an association of ASIC and MDEG was obtained from co-immunoprecipitation experiments. ASIC and MDEG were tagged with short epitopes (“T7tag\(^{TM}\) and “Flag\(^{TM}\)”) introduced at their NH\(_2\) terminus. The tagged proteins ASIC-tag and MDEG-Flag were expressed either alone or coexpressed in SF9 cells using a baculovirus system. Electrophysiological analysis of the infected cells was carried out to show that the introduction of the epitope does not alter the channel properties (data not shown). The detergent solubilized membranes of SF9 cells expressing either ASIC-tag or MDEG-Flag or both subunits were immunoprecipitated with an anti-Flag(MDEG) antibody, and both the immunoprecipitated fractions and the supernatants were analyzed by Western blot. Analysis of the blots with the anti-Flag(MDEG) antibody revealed that at least 50% of MDEG-Flag was immunoprecipitated (lane 2) and that the anti-Flag(MDEG) antibody did not cross-react with ASIC-tag (lane 1). We then incubated the same blots with an anti-tag(ASIC) antibody (Fig. 1B, bottom panel) to analyze whether immunoprecipitation of MDEG-Flag caused co-precipitation of ASIC-tag. The anti-tag(ASIC) antibody did not cross-react with MDEG-Flag, since a signal was only obtained with samples that contained ASIC-tag. When both ASIC-tag and MDEG-Flag were co-expressed in the same cells, immunoprecipitation with the anti-Flag(MDEG) antibody caused co-precipitation of ASIC-tag (lane 3), indicating that ASIC-tag associates with MDEG-Flag to form a heteromultimeric channel. The heteromultimeric association occurs in vivo and not after solubilization of the cells. No co-immunoprecipitation of ASIC-tag by the anti-Flag(MDEG) antibody was found when we used a mixture of
FIG. 2. Biophysical and pharmacological properties of ASIC + MDEG coexpressed in oocytes. Currents were recorded from outside-out patches from Xenopus oocytes at 0 mV. A, current responses induced by different pH changes. B, pH dependence of ASIC/MDEG (△). Points represent mean values from 5 to 11 outside-out patches. The pH dependencies of ASIC and MDEG are given for comparison. The dashed line represents the theoretical pH dependence of a mixture of equivalent amounts of ASIC and MDEG in the absence of any interaction between them. C and D, effect of amiloride on the proton-gated current induced by a pH drop from pH 7.4 to pH 4. D, points represent mean values from five experiments. E, histogram of the mean inactivation time constants of the inward current induced by pH drops from 7.4 to 4 at 0 mV. F, semilogarithmic plot of H⁺-gated currents desactivation illustrating the single exponential decay. Currents were normalized to the value at t = 0.

membranes from cells expressing either ASIC-tag or MDEG-Flag (lane 4, bottom panel).

Both ASIC (4) and MDEG (5) expressed in Xenopus laevis oocytes form amiloride-sensitive H⁺-gated cation channels that are activated transiently when the extracellular pH drops (4). The ASIC and the MDEG channels have different inactivation kinetics, ion selectivities, and pH dependencies (Figs. 2, B, E, and F, and 3E). Coexpression of both ASIC and MDEG yields an amiloride-sensitive H⁺-gated current (Fig. 2A) with single exponential inactivation kinetics that are intermediate between those of the ASIC and the MDEG current (Fig. 2, E and F) instead of the biphasic kinetics that would be expected for two independent homomultimeric channels. The pH dependence of the H⁺-activated current obtained after coexpression of ASIC and MDEG (Fig. 2B) is less steep than with either subunit alone and the pH at which half-maximal activation occurs (pH_{0.5} = 6.2) or MDEG (pH_{0.5} = 4.1) alone. Besides, the pH dependence curve of the ASIC/MDEG channel is monophasic, while a biphasic curve is expected for independent equimolar expression of the two subunits (Fig. 2B, dashed line). The channel formed after coexpression of ASIC and MDEG is less selective for Na⁺ versus K⁺ than either the ASIC or the MDEG channel (Fig. 3, A, B, and E), suggesting that most of the channel activity recorded in oocytes expressing both ASIC and MDEG is due to a heteromultimeric channel. Amiloride blocks the ASIC/MDEG channel as it blocks ASIC and MDEG activity (Fig. 2, C and D). The K_{50} value for this inhibition is 20 μM. The single channel conductance of the ASIC/MDEG channel is 10 pS (Fig. 3, C and D).

Substitution of an alanine just before the second putative transmembrane domain of the degenerin MEC-4 of the nematode C. elegans by bulky amino acids, such as phenylalanine or valine, causes degeneration of mechanosensitive neurons (23). This phenotype was proposed to be due to a gain-of-function of a putative stretch-activated channel composed of three subunits, MEC-4, MEC-6, and MEC-10 (19). Mutation of the corresponding amino acid (Gly^{431} in MDEG) also causes constitutive activity and cell death (5). We initially reported that identical mutations (Gly^{431} → Val or → Phe) introduced into ASIC do not cause constitutive channel activity (4). However a more exhaustive study of the amiloride-insensitive basal currents of ASIC Gly^{431} mutants revealed a low constitutive channel activity for G431V or a larger activity for the G431F mutant (Fig. 4, A and B). These constitutive channels were not inhibited by 1 mM amiloride (not shown) and have a reversal potential (V{rev}) of +30 mV for G431V and +15 mV for the G431F mutant (Fig. 4B). Responses to external H⁺ were also markedly altered by mutations. Increases in H⁺ concentrations did not induce any channel activation of the G431V mutant and they decreased the amplitude of the constitutive current of the G431F mutant (Fig. 4D). ASIC(G431V) and ASIC(G431F) were found to greatly alter the properties of MDEG (Fig. 4, C, D, and E). Coexpression of the ASIC mutant G431V with MDEG in X.
ASIC and MDEG Form a Heteromultimeric $H^+$-gated Cation Channel

Figure 4. Effect of ASIC mutations on the activity of the ASIC and ASIC/MDEG channel. A, histogram of the mean basal currents at pH 7.4 measured at -70 mV. B and C, mean I-V relationships of the whole oocyte currents measured by voltage ramps from -150 mV to +100 mV at pH 7.4. D, current responses induced by a drop of pH from 7.4 to 5 recorded from whole oocytes. E, pH dependence of the $H^+$-activated ASIC(G431V)/MDEG and ASIC(G431F)/MDEG currents. Points represent mean values from 15 oocytes. The added curve indicates the pH dependence of MDEG for comparison.

Laevos oocytes induced an amiloride-insensitive nonselective current (Fig. 4, A and C) with an intensity which is much higher than the sum of MDEG or ASIC(G431V) currents (Fig. 4A), indicating that a novel heteromultimeric channel has been formed by association of both subunits. Extracellular acidification from pH 7.4 to below pH 7 further activated the ASIC(G431V)/MDEG (pH 0.5 = 6.2) or ASIC(G431F)/MDEG (pH 0.5 = 5.8) channels (Fig. 4, D and E). Since the mutant subunits ASIC(G431V) and ASIC(G431F) cannot form proton-activated channels by themselves, and since the activation of MDEG requires an extracellular acidification to pH values lower than 5 (Fig. 4E), the proton activated current recorded at pH values above pH 5.5 in oocytes co-expressing ASIC(G431V) or ASIC(G431F) together with MDEG can only be explained by the formation of a heteromultimeric channel.

Like many other ligand-gated cation channels, such as the ionotropic glutamate and purinergic receptors (11), ASIC as well as MDEG subunits can form both homomultimeric and heteromultimeric channels. The formation of heteromultimeric channels with novel properties augments the repertoire of $H^+$-gated channels and introduces new possibilities of regulation. For example, ASIC displays a consensus site for protein kinase A phosphorylation in position 474 and two consensus sites for protein kinase C phosphorylation in positions 40 and 497. Association with MDEG introduces a new consensus site for protein kinase C in position 39.

Interestingly $H^+$-gated cation currents with kinetics and pH dependencies similar to those of the heteromultimeric ASIC/MDEG channel were recorded in brain neurons (24). Rapid acidic pH transients accompany synaptic activity and were proposed to be involved in neuromodulation (25, 26). It is thus tempting to speculate that $H^+$-gated cation channels present in neurons, such as ASIC and MDEG, but also the heteromultimeric ASIC/MDEG channels, have a mediating role between extracellular acidic transients and changes in neuronal excitability.

Acknowledgments—We are very grateful to Dr. Eric Lingueglia for helpful discussions and to Catherine Le Calvez, Catherine Widman, Gisèle Jarretou, Martine Jodar, and Nathalie Leroudier for their skillful technical assistance, Dalvyda Doume for secretarial assistance, and Frank Aguila for help with the artwork.

REFERENCES
1. Bevan, S., and Yeats, J. (1991) J. Physiol. (Lond.) 433, 145–161
2. Akaie, N., and Ueno, S. (1994) Prog. Neurobiol. 43, 73–83
3. Krishtal, O. A., and Pidoplichko, V. I. (1981) Neuroscience 6, 2599–2601
4. Waldmann, R., Champigny, G., Bassilana, F., Heurteaux, C., and Lazdunski, M. (1997) Nature 386, 173–177
5. Waldmann, R., Champigny, G., Voilley, N., Lauritzen, I., and Lazdunski, M. (1996) J. Biol. Chem. 271, 10433–10434
6. Price, M. P., Snyder, P. M., and Walsh, M. J. (1996) J. Biol. Chem. 271, 7879–7882
7. Chaffie, M., and Wolinsky, E. (1990) Nature 345, 410–416
8. Driscoll, M., and Chaffie, M. (1991) Nature 349, 588–593
9. Chalfie, M., and Wolinsky, E. (1990) Nature 349, 588–593
10. Davies, N. W., Lux, H. D., and Morad, M. (1988) J. Physiol. (Lond.) 400, 159–187
11. Barnard, E. A. (1996) Trends Pharmacol. Sci. 17, 305–309
12. Canessa, C. M., Schild, L., Buell, G., Thorens, B., Gautschi, I., Horisberger, J. D., and Rossier, B. C. (1994) Nature 367, 463–467
13. Canessa, C. M., Horisberger, J. D., and Rossier, B. C. (1993) Nature 361, 467–470
14. Lingueglia, E., Renard, S., Waldmann, R., Voilley, N., Champigny, G., Plass, H., Lazdunski, M., and Barbery, P. (1994) J. Biol. Chem. 269, 13736–13739
15. Lingueglia, E., Voilley, N., Waldmann, R., Lazdunski, M., and Barbery, P. (1993) FEBS Lett. 318, 95–99
16. Waldmann, R., Champigny, G., Bassilana, F., Voilley, N., and Lazdunski, M. (1995) J. Biol. Chem. 270, 27411–27414
17. Garty, H., and Palmer, L. G. (1997) Physiol. Rev. 77, 359–396
18. Garty, H. (1994) FEBS Lett. 352, 522–528
19. Huang, M., and Chaffie, M. (1994) Nature 367, 467–470
20. Gu, G., Caldwell, G. A., and Chaffie, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6577–6582
21. Herman, R. K. (1996) Biosci. Rep. 16, 199–206
22. Garcia-Aianoveros, J., Derfler, B., Neville-Golden, J., Hyman, B. T., and Corey, D. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1459–1464
23. Hong, K., and Driscoll, M. (1994) Nature 367, 470–473
24. Ueno, S., Nakaye, T., and Akaie, N. (1992) J. Physiol. (Lond.) 447, 309–327
25. Krishtal, O. A., Osipchuk, Y. V., Shelest, T. N., and Smirnoff, S. V. (1987) Brain Res. 406, 352–356
26. Chaffie, M. (1990) Prog. Neurobiol. 34, 401–427