dGNaC1, a Gonad-specific Amiloride-sensitive Na⁺ Channel*

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Amiloride-sensitive sodium channels have been implicated in reproductive and early developmental processes of several species. These include the fast block of polyspermy in Xenopus oocytes that follows the sperm binding to the egg or blastocoel expansion in mammalian embryo. We have now identified a gene called dGNaC1 that is specifically expressed in the gonads and early embryo in Drosophila melanogaster. The corresponding protein belongs to the superfamily of cationic channels blocked by amiloride that includes Caenorhabditis elegans degenerins, the Helix aspersa FMRF-amide ionotropic receptor (FaNaC), the mammalian epithelial Na⁺ channel (ENaC), and acid-sensing ionic channels (ASIC, DRASIC, and MDEG). Expression of dGNaC1 in Xenopus oocytes generates a constitutive current that does not discriminate between Na⁺ and Li⁺, but is selective for Na⁺ over K⁺. This current is blocked by amiloride (IC₅₀ = 24 μM), benzamil (IC₅₀ = 2 μM), and ethylisopropyl amiloride (IC₅₀ = 49 μM). These properties are clearly different from those obtained after expression of the previously cloned members of this family, including ENaC and the human αENaC-like subunit, δNaC. Interestingly, the pharmacology of dGNaC1 is not very different from that found for the Na⁺ channel characterized in rabbit preimplantation embryos. We postulate that this channel may participate in gametogenesis and early embryonic development in Drosophila.

The importance of amiloride-sensitive sodium transport during reproductive and early developmental processes is highlighted by numerous experiments performed in diverse organisms, such as sea urchins (1, 2), fish (3), amphibians (4–6), and mammals (7). Amiloride interacts not only with ubiquitous Na⁺/H⁺ antiporters, which are also expressed in gonads and are involved in initiation of development at fertilization (8), but also with amiloride-blockable channels that are involved in the fast block of polyspermy (9) and blastocoel expansion (10). In Xenopus oocytes, ATP triggers an amiloride-sensitive Na⁺ channel immediately after the sperm has bound to the egg. This depolarizes the egg membrane potential and participates in the fast block of polyspermy (9). In mammalian embryo, amiloride-sensitive sodium channels are also involved in the blastocoel expansion (11). During early phases of animal embryonic development, blastomeres progressively occupy the periphery of the embryo, and a fluid-filled central cavity, i.e. the blastocoel, is formed. Fluid accumulation in the blastocoel is due to an electrogenic transport of sodium, followed by osmotically driven water, and this event can be partly inhibited by amiloride (10, 12, 13). Electrophysiological analyses have demonstrated that amiloride-sensitive channels are expressed in rabbit blastomeres, but their biophysical and pharmacological properties differ from those of the classical highly Na⁺-selective and highly amiloride-inhibitable channel (10).

Amiloride sensitivity is a common characteristic of structurally related cationic channels that are associated with a wide range of distinct physiological functions (14). In Caenorhabditis elegans, neuronal and muscular degenerins, such as MEC-4, MEC-10, UNC-8, and UNC-105, encode amiloride-sensitive channels that are involved in mechanoperception (15–17). In animal epithelia, a highly sodium-selective channel is made up of three homologous subunits (αENaC (epithelial Na⁺ channel), βENaC, and γENaC) (18–21). This channel participates in active vectorial sodium transport. In the snail nervous system, FaNaC is an ionotropic receptor for the mollusc cardioexcitatory peptide FMRF-amide. It forms a homotetrameric sodium-selective channel that may be involved in neuromodulation (22, 23). In mammalian brain and/or in sensory neurons, acid-sensing ionic channels (ASIC) are homo- or heteromultimeric H⁺-activated cation channels (24–27). They are suspected to be involved in nociception linked to acidosis. All these proteins share the same structural organization, characterized by the presence of two hydrophobic domains surrounding a large extracellular loop that includes one cysteine-rich region (or two for degenerins) (28). Despite a very low identity between the most distantly related proteins of the family (~15%), some important residues, such as those located in pre-M1, pre-M2, and M2 regions (where M1 and M2 represent the two transmembrane hydrophobic α-helices), are conserved.

Different members of this family have been described in mammalian gonads or in adjacent tissues. Expressed sequence tags of αENaC, δNaC (Na⁺ channel δ subunit; an αENaC-like subunit) (29), and ASIC-1 have been identified in human testis. Only human δNaC was indeed detected in testis and ovary by Northern blot analysis (29). This raises the possibility that members of the degenerin/ENaC/FaNaC/ASIC gene superfamily might be involved in the differentiation of gametes and/or development of eggs after fertilization.

In this study, we have characterized a new member of the family in D. melanogaster, which is specifically expressed in testis, ovary, and early embryo. Its expression in Xenopus oocytes was sufficient to generate a constitutive amiloride-sensitive Na⁺ channel, with properties that differ from those obtained after expression of αβγENaC or δNaC/βγENaC. Since this Drosophila Na⁺-selective channel is specifically expressed in the gonads and early embryo, we postulate that it may

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An Amiloride-sensitive Na\(^+\) Channel in Drosophila Gonads

Correspond to a channel involved in spermatogenesis, oogenesis, and/or early embryonic development.

**EXPERIMENTAL PROCEDURES**

**Cloning of dGNaC1—** Two sequences from the Drosophila expressed sequence tag data base (accession numbers AA264333 and AA264288) were used to design a sense and an antisense primer carrying an EcoRI (sense primer) and an XhoI (antisense primer) restriction site, respectively. The corresponding sequences are as follows:

5'ACGAATTCAAGAACGGAATAGACACCATG-3'

and

5'TCCTCGAGTTGTCCGCACTTACGAAATAG-3'.

These primers were used to amplify a 1750-base pair fragment by PCR with the Expand High Fidelity PCR system (Boehringer Mannheim) from Drosophila cDNAs prepared from mid-stage embryos. After methylation of the internal EcoRI restriction site with EcoRI methylase (Biolabs), ligation of EcoRI linkers (Biolabs), and digestion by EcoRI and XhoI, the fragment was subcloned in the EcoRI and XhoI sites of the pBSK-SP6-globin vector (30). Three independent clones were sequenced on both strands. They display an open reading frame of 1686 nucleotides that is not preceded by stop codon.

**Fly Stocks—** All fly stocks (Oregon R or w1118) were maintained under standard culture conditions.

**In Situ Chromosomal Mapping—** For in situ hybridization to polytene salivary gland chromosomes, the pBSK-SP6-globin vector containing the entire cDNA sequence of dGNaC1 was labeled with biotin-11-dUTP (Boehringer Mannheim) by random priming according to the manufacturer's instructions.

**Expression in Oocytes and Electrophysiological Analysis—** For expression in Xenopus oocytes, cRNA was synthesized from the NotI-digested vector using a kit from Stratagene. Xenopus oocytes were
injected with 0.5–5 ng of cRNA, and microelectrode voltage-clamp assays were performed 1–5 days after injection.

Northern Blotting and RT-PCR—Total RNA was obtained from staged embryos, larvae, pupae, and adults by the acidic phenol method (31). Five micrograms of total RNA from each developmental stage were fractionated by electrophoresis on a formaldehyde-containing 0.8% agarose gel and transferred to Nylon membrane (Hybond N, Amersham Pharmacia Biotech). The entire coding sequence of dGNaC1 was radio-labeled by the random priming method (Promega) and used as a probe (10⁶ cpm/ml) for overnight hybridization at 42 °C in a solution containing 30% formamide, 5× Denhardt’s solution, 5× SSC, 0.1% SDS, and 100 µg/ml denatured salmon sperm DNA. After washing in 0.2× SSC and 0.1% SDS at 55 °C, the blot was exposed to Kodak X-Omat AR film. The filter was subsequently hybridized with a probe encoding the Drosophila rpl17 gene (32) to estimate the relative amount of RNA loaded in each lane.

For RT-PCR experiments, total RNAs were treated with RNase-free DNase I (Boehringer Mannheim) to remove contaminating genomic DNA. For the first strand cDNA synthesis, 2 µg of total RNA were oligo(dT)-primed in a final volume of 20 µl in the presence or absence of 200 units of Superscript II reverse transcriptase (Life Technologies, Inc.). The entire coding sequence of dGNaC1 was radio-labeled by the random priming method (Promega) and used as a probe (10⁶ cpm/ml) for overnight hybridization at 42 °C in a solution containing 30% formamide, 5× Denhardt’s solution, 5× SSC, 0.1% SDS, and 100 µg/ml denatured salmon sperm DNA. After washing in 0.2× SSC and 0.1% SDS at 55 °C, the blot was exposed to Kodak X-Omat AR film for 14 days at −70 °C with intensifying screens. The filter was subsequently hybridized with a probe encoding the Drosophila rpl17 gene (32) to estimate the relative amount of RNA loaded in each lane.

RESULTS

Cloning and Structure of dGNaC1—Two partial cDNA sequences from Drosophila embryo similar to those of other members of the degenerin/ENaC/FaNaCASIC superfamily were found in the data base of expressed sequence tags (accession numbers AA264333 and AA264288). One displayed similarity in the 5′-coding region (AA264333), including the first transmembrane domain, and contained a putative start codon. The other (AA264288) showed similarity in the 3′-coding region, including the second transmembrane domain, and contained a putative stop codon. Two oligonucleotides flanking the putative coding sequence were used to amplify by PCR a fragment of 1750 base pairs from Drosophila cDNA. It contains an open reading frame of 1686 base pairs and codes for a protein of 562 amino acids (Fig. 1A). This protein has all the hallmarks of the degenerin/ENaC/FaNaCASIC superfamily, i.e. two hydrophobic domains flanking a large region including a cysteine-rich domain (Fig. 1, A and B) that was shown to be extracellular for the epithelial Na⁺-channel (34) and for the degenerin MEC-4 (35). dGNaC1 was mapped by in situ hybridization to region 82CD on the right arm of the third chromosome (data not shown).

Expression of dGNaC1 in Xenopus Oocytes—When dGNaC1 cRNA was injected into Xenopus oocytes, an amiloride-sensitive Na⁺-selective current was recorded (Fig. 2A). Large variations in the amplitude of the amiloride-sensitive current were found in different oocyte batches (from a few tens of nA up to ~1 µA). Treatment with ATP, a jump in the external pH, and activation of protein kinases A and C were not able to alter the current (data not shown). The ionic substitutions experiments (Fig. 2C) and the inversion of the amiloride-sensitive current at positive potential values (Eᵢ = 36.5 ± 6.6 mV, n = 11) (Fig. 2D) suggested a higher permeability of the channel for Na⁺ over K⁺ and an equal permeability for Li⁺ and Na⁺. Amiloride and its derivative, ethylisopropyl amiloride, blocked the channel, with half-inhibition concentrations (IC₅₀) of 24 and 49 µM, respectively, whereas benzamil was found to be more effective, with an IC₅₀ of 2 µM (Fig. 2B).
Expression of dGNaC1 during Development—The expression pattern of dGNaC1 was analyzed by Northern blot hybridization of total RNA isolated from different developmental stages (Fig. 3A). Three observations were made. First, two forms of mRNA are transcribed: one major RNA transcript of 3.2 kb and a less abundant transcript of 2.3 kb, probably explained by different mRNA polyadenylation, alternative splicing of the dGNaC1 gene, or the presence of a closely related homologous mRNA. Second, the expression level is strongly regulated during development (Fig. 3A). The mRNA was readily detected in early embryos (0–4 h), suggesting a high level of maternal expression. However, no further expression was detected during late embryogenesis. Third, dGNaC1 is specifically transcribed in the gonads (Fig. 3, A and B). The highest RNA level was detected in ovaries; mRNA was still detectable in whole females, but not in females lacking ovaries or in males (Fig. 3A). More sensitive measurements made using RT-PCR detected dGNaC1 transcripts in whole males due to specific expression in the genital tract (Fig. 3B). No signal was observed in males after removal of their genital tracts or in females after removal of their ovaries (Fig. 3B).

A more precise determination of dGNaC1 transcript localization was assessed by in situ hybridization. Experiments with digoxigenin-labeled antisense RNA probes and control experiments with sense probes were performed on wild-type ovaries and testes (Fig. 4). dGNaC1 RNA was not detectable in ovarian stem cells, oogonia, and early cysts (Fig. 4A). They were weakly detected at stage 5 (according to the criteria of King (36)), and the highest expression was seen at stage 10, where they were present throughout the nurse cells, oocytes, and follicle cells (Fig. 4, A and B). In situ hybridization was also performed on whole-mount embryos. The criteria of Campos-Ortega and Hartenstein (37) were used to identify stages of embryonic development. At the syncytial stage (stage 4), prior to cellularization, dGNaC1 was distributed uniformly within the cytoplasm (Fig. 4C). It was also detected in the cellular blastoderm embryo (Fig. 4D). The pole cells, the progenitors of the germ line, did not accumulate dGNaC1 transcripts (Fig. 4D). Levels of transcript then declined in embryos at stage 8, i.e. at early gastrulation (Fig. 4E), and subsequently vanished.

In situ hybridization on the male genital tract also revealed the presence of transcript. dGNaC1 was not detected in the apical tips, which contain the stem cells and gonial cysts, but it was detected in the cysts that contain primary spermatocytes or cells entering meiosis (Fig. 4G). dGNaC1 was also detected in seminal vesicles (Fig. 4G).

DISCUSSION

This work reports the identification of a gene related to the degenerin/ENaC/FaNaC/ASIC superfamily of ion channels in D. melanogaster. This finding is consistent with the view that amiloride-sensitive Na⁺ channels are widely expressed throughout the whole animal kingdom. Physiological studies have previously shown that a channel related to the epithelial sodium channel is present in the leech Hirudo medicinalis, where it controls animal volume (38), and in Lumbricus terrestris intestine, where amiloride-sensitive sodium transport displays seasonal changes via an unknown hormonal regulatory mechanism (39). Moreover, the C. elegans degenerins are widely expressed in nematode neurons and muscle (40), and Helix or Aplysia neurons express the FMRF-amide ionotropic receptor (22).

The Drosophila gene encodes dGNaC1, a protein of 562 amino acids that contains all the conserved motifs characteristic of the family. This protein has significant but low sequence identity to the other members of the family (below 20%) (Fig. 1C) and cannot be linked to any of them by phylogenetic analyses (data not shown), except for another protein identified in Drosophila peripheral nervous system that displays 38% identity to dGNaC1 (41, 53). Similarity was stronger around the first and second transmembrane domains, but was low in the large extracellular loop, except for the cysteine-rich region and the two highly conserved motifs 107FFAVTVC113 and 224GI-CYTFTN230 (Fig. 1A). Despite distant phylogenetic relationships, the overall structure of dGNaC1 makes it closer to αENaC, βENaC, γENaC, or δNaC than to the other members of the family (Fig. 1B). According to the classification proposed by Barbry and Hofman (28), dGNaC1 would therefore group with the proteins involved in vescicular transport, in agreement with the functional properties of the dGNaC1 channel recorded in Xenopus oocytes (Fig. 2). It should be noted that the intracellular COOH-terminal part of dGNaC1, which was shown to have an important role in epithelial sodium channel regulation (42–44), is extremely short.

The channel expressed in Xenopus oocytes after injection of the in vitro transcribed RNA is highly selective for Na⁺ over K⁺ and is blocked by amiloride. While ENaC is more permeant to Li⁺ than to Na⁺, dGNaC1 does not discriminate between Na⁺ and Li⁺ and displays a small but significant K⁺ permeability. Amiloride concentrations needed to block this channel are 10–100 times higher than those necessary to block ENaC or δNaC (20, 29). Ethylisopropyl amiloride, a classical high affinity blocker of the Na⁺/H⁺ antiporter, can also efficiently block the
dGNaC1 channel, with a potency similar to that of amiloride. Ethylisopropyl amiloride is much less active than amiloride in blocking the ENaC channel (20). The dGNaC1 single-channel conductance was difficult to assess due to a noisy current with no resolved unitary current levels (data not shown). The pharmacological and biophysical properties of dGNaC1 are very close to those of an αβγENaC channel comprising a mutated α-subunit in which Ser 589 in the second transmembrane domain was changed to a phenylalanine (30), i.e. the residue found in the equivalent position of dGNaC1.

Successful heterologous expression of dGNaC1 was obtained only in Xenopus oocytes. It was actually not possible to record any dGNaC1 activity in transfected mammalian COS cells (data not shown). This strongly suggests that dGNaC1 channel activity is modulated by a Xenopus oocyte-specific factor. This is particularly noteworthy since expression of dGNaC1 transcripts in Drosophila was also restricted to oocytes of late vitellogenic stages and to early embryos, in addition to nurse cells and follicular cells. Transcripts disappeared completely at the stage of late gastrulation. Thus, dGNaC1 corresponds to a maternally encoded gene.

The functional properties of dGNaC1 described here can presently be related to two distinct physiological processes. First, between stages 10 and 14, the oocyte develops in an egg chamber comprising a cyst of 15 nurse cells interconnected by ring canals at the anterior edge. The single oocyte is surrounded with an epithelium layer of follicle cells (for review, see Ref. 45). Active transcription and translation by nurse cells produce the different constituents that are necessary for efficient growth of the oocyte. They are coupled to transport from nurse cells to the oocyte through intercellular junctions. Voltage gradients between nurse cells and the oocyte were first reported and proposed to explain this transport by Woodruff and Telfer (46). However, available evidence does not support a role for electrophoresis in the early phases of transport (47). Using a vibrating probe, Overall and Jaffé (48) have described a large steady Na⁺ influx through the anterior or nurse cell end of the follicles. Coupled with efflux at the posterior side of the egg, this transcellular transport is expected to be coupled to osmotic transport of water. The drag effect that can accompany this active Na⁺ transport would in that case carry macromolecules. dGNaC1 transcripts were found in nurse cells and follicular cells. This raises the possibility that dGNaC1 is involved in the entry of sodium into nurse cells. A second potential role for dGNaC1 could be in the hydration event that is associated with ovulation in Drosophila (49) and more generally in the large swelling of the oocytes that is observed during final maturation. Interestingly, LaFleur and Thomas (3) have reported that in marine teleosts, such oocyte swelling is partially blocked by amiloride (3).

A similar dGNaC1 function related to volume increase could take place in testis, where dGNaC1 transcripts are detected in the primary spermatocyte stage of development and in later cells in the spermiogenic pathway. The primary spermatocyte transcribes most if not all of the gene products needed for the dramatic morphogenetic events that follow meiosis (for review, see Fuller (50)). The primary spermatocyte stage lasts 90 h. During this time, the cells grow 25 times in volume (51). Such volume increase may be related to electrogenic sodium transport through dGNaC1, osmotically followed by water. Nevertheless, since there exist mechanisms that act during spermatogenesis to delay translation of certain messages until well after transcription, a function later in spermiogenesis cannot be excluded.

Amiloride-sensitive Na⁺ channels have been implicated in vertebrate development. In mammalian embryo, the blastocoele is formed by fluid accumulation due to an electrogenic transport of sodium partly inhibited by amiloride (10, 12, 13). In Drosophila, a stage 5 embryo, which corresponds to mammalian blastula, is indeed a syncytium lacking a fluid-filled central cavity (for review, see Foe et al. (52)). Thus, dGNaC1 can hardly be linked to blastocoele expansion. Nevertheless, the Na⁺ channel characterized in 7-day postcoitus preimplantation embryos in rabbits is inhibited by amiloride, benzamil, and ethylisopropyl amiloride, with apparent dissociation constants of 12, 50, and 16 μM, respectively (10). These values are not very different from those found for dGNaC1, but they are clearly distinct from those found for the classical epithelial Na⁺ channel (28) or for the human sodium channel δ-subunit (29).

One can thus infer from the gonad-specific expression of
dGNaC1 in *Drosophila* that new members of the degenerin/ENaC/FaNaC/ASIC gene superfamily similar to dGNaC1 will be identified in vertebrate gonads, where they will be involved in early developmental processes.

In conclusion, we report here the properties of dGNaC1, a new gonad-specific *Drosophila* amiloride-sensitive Na⁺ channel that may participate in gametogenesis and early embryonic development. Elucidation of the regulatory properties of this channel will probably reveal important mechanisms controlling early steps of development.

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Note Added in Proof—dGNaC1 is identical to the Ripped Pocket (RPK) protein recently described by Adams et al. (53).

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