



Molecular characterization and functional expression of the *Apis mellifera* voltage-dependent Ca²⁺ channels



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ABSTRACT

Voltage-gated Ca²⁺ channels allow the influx of Ca²⁺ ions from the extracellular space upon membrane depolarization and thus serve as a transducer between membrane potential and cellular events initiated by Ca²⁺ transients. Most insects are predicted to possess three genes encoding Cav α , the main subunit of Ca²⁺ channels, and several genes encoding the two auxiliary subunits, Cav β and Cav α 2 δ ; however very few of these genes have been cloned so far. Here, we cloned three full-length cDNAs encoding the three Cav α subunits (AmelCav1a, AmelCav2a and AmelCav3a), a cDNA encoding a novel variant of the Cav β subunit (AmelCav β c), and three full-length cDNAs encoding three Cav α 2 δ subunits (AmelCav α 2 δ 1 to 3) of the honeybee *Apis mellifera*. We identified several alternative or mutually exclusive exons in the sequence of the AmelCav2 and AmelCav3 genes. Moreover, we detected a stretch of glutamine residues in the C-terminus of the AmelCav1 subunit that is reminiscent of the motif found in the human Cav2.1 subunit of patients with Spinocerebellar Ataxia type 6. All these subunits contain structural domains that have been identified as functionally important in their mammalian homologues. For the first time, we could express three insect Cav α subunits in *Xenopus* oocytes and we show that AmelCav1a, 2a and 3a form Ca²⁺ channels with distinctive properties. Notably, the co-expression of AmelCav1a or AmelCav2a with AmelCav β c and AmCav α 2 δ 1 produces High Voltage-Activated Ca²⁺ channels. On the other hand, expression of AmelCav3a alone leads to Low Voltage-Activated Ca²⁺ channels.

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Abbreviations: VGCC, Voltage-Gated Ca²⁺ Channel; HVA, High Voltage-Activated; LVA, Low Voltage-Activated; ORF, Open Reading Frame; RACE, Rapid Amplification of cDNA Ends; AID, Alpha Interaction Domain; GPI, glycosyl-phosphatidylinositol.

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1. Introduction

Ca²⁺ ions are a widespread second messenger that participates in many cellular functions. The flux of Ca²⁺ ions through Voltage-Gated Ca²⁺ Channels (VGCC) is involved in excitation-secretion coupling in neurons and endocrine cells, excitation-contraction coupling in muscular cells and excitation-transcription coupling (reviewed in Catterall, 2011; Bellis et al., 2013). In honeybees, Ca²⁺ influx participates in the formation and consolidation of the olfactory memory (see for review Sandoz, 2011). However, the precise role played by VGCCs in olfactory memory is difficult to evaluate without specific blockers. VGCCs open through plasma membrane depolarization, a process called activation, to allow the

influx of ions from the extracellular space. Then, during persistent depolarization, they undergo inactivation and closure to prevent cytoplasmic Ca^{2+} overload. VGCCs are hetero-multimers composed of one $\text{Cav}\alpha$ subunit (the pore-forming subunit) that may be associated with auxiliary subunits, $\text{Cav}\beta$ and $\text{Cav}\alpha 2\delta$ (Fig. 1). The $\text{Cav}\alpha$ subunit carries most of the molecular apparatus that governs channel gating (opening and closure). The auxiliary subunits can profoundly modify channel gating and therefore deeply affect the properties of Ca^{2+} currents. Specifically, the $\text{Cav}\beta$ subunit increases $\text{Cav}\alpha$ sensitivity to voltage, thus hyperpolarizing the current–voltage curve, and speeds up or slows down channel inactivation (see for review Buraei and Yang, 2010). The $\text{Cav}\alpha 2\delta$ subunit also shapes the biophysical properties of $\text{Cav}\alpha$, but less extensively than $\text{Cav}\beta$ (Dolphin, 2013). They both contribute to the trafficking and membrane stabilization of the channel (Felix et al., 2013). In mammals, where Ca^{2+} channels have been extensively studied, VGCCs are divided in two classes: Low Voltage-Activated (LVA) and High Voltage-Activated (HVA) channels that require low or high depolarization to open, respectively (Catterall, 2011). This phenotypic differentiation relies on the existence of different $\text{Cav}\alpha$ subunit families. Indeed, the Cav1 and Cav2 subunits give rise to HVA channels and the Cav3 subunits to LVA channels. Mammalian genomes contain four genes encoding four Cav1 subunits (Cav1.1 to 1.4), three genes encoding three Cav2 subunits (Cav2.1 to 2.3) and three genes encoding three Cav3 subunits (Cav3.1 to 3.3). These different $\text{Cav}\alpha$ subunits are structurally and functionally related, but have specific electrophysiological and pharmacological properties. Thus, Cav1 subunits give rise to L-type Ca^{2+} channels, Cav2 subunits to P/Q, N and R type Ca^{2+} channels, and Cav3 subunits to T-type Ca^{2+} channels (Catterall, 2011). While auxiliary subunits ($\text{Cav}\beta$ and $\text{Cav}\alpha 2\delta$) are integral members of channel hetero-multimers formed with Cav1 or Cav2, there is little evidence for their association with Cav3. In mammals, there are four genes encoding $\text{Cav}\beta$ ($\text{Cav}\beta 1$ to 4) and four genes encoding $\text{Cav}\alpha 2\delta$ subunits ($\text{Cav}\alpha 2\delta 1$ to 4). As these auxiliary subunits finely tune $\text{Cav}\alpha$ properties, these multiple subunit combinations considerably increase the Ca^{2+} channel repertoire. Moreover, the presence of alternative exons in the coding sequence of all these subunits adds another layer of complexity. In invertebrates, including insects, things appear to be less complex because a single gene for the Cav1, Cav2 and Cav3 families has been identified and usually there is also a single gene

for $\text{Cav}\beta$, but several genes for $\text{Cav}\alpha 2\delta$ (Tyson and Snutch, 2013). Very few Ca^{2+} channel subunits have been cloned in invertebrates (Bouchard et al., 2006; Jeziorski et al., 1998; Kimura and Kubo, 2003, 2002; Kohn et al., 2001; Smith et al., 1998; Zheng et al., 1995) with the notable exception of the three $\text{Cav}\alpha$ and one $\text{Cav}\beta$ of the pond snail *Lymnaea stagnalis* (Dawson et al., 2014; Senatore and Spafford, 2010; Spafford et al., 2003, 2006). This is certainly explained by the difficulty to express invertebrate $\text{Cav}\alpha$ subunits in the heterologous expression systems commonly used for mammalian Ca^{2+} channels, such as *Xenopus* oocytes or HEK293 cells (Salvador-Recatalà and Greenberg, 2012). Moreover, arthropod and mammalian Ca^{2+} currents display different pharmacological sensitivities and compounds commonly used in mammals to specifically discriminate the different types of Ca^{2+} channels cannot be easily used in insects (Jeziorski et al., 2000).

The genome of the honeybee *Apis mellifera* contains one gene for each $\text{Cav}\alpha$ family, a single gene for $\text{Cav}\beta$ and three genes for $\text{Cav}\alpha 2\delta$ (Weinstock et al., 2006). In a previous work, we reported the cloning of two $\text{Cav}\beta$ splice variants and their functional expression with a mammalian $\text{Cav}\alpha$ subunit (Cens et al., 2013). Here, we describe the molecular characterization of a third $\text{Cav}\beta$ splice variant, of the three $\text{Cav}\alpha$ and the three $\text{Cav}\alpha 2\delta$ subunits. We show that these subunits contain molecular domains that are functionally important for the electrophysiological properties of Ca^{2+} channels. We also describe for the first time the functional expression of insect VGCCs and demonstrate that the three $\text{Cav}\alpha$ subunits lead to distinctive Ca^{2+} currents. The heterologous expression of honeybee VGCCs will not only allow the characterization of the biophysical properties of these channels but also the assessment of specific blockers that would be used in intact honeybee cells.

2. Material and methods

2.1. Tissue preparation

Pupae and adult worker bees were anaesthetized by chilling at 4 °C. Heads, legs, abdomens, antenna were collected and stored on ice in TRIzol[®] Reagent (Life Technologies). Whole brain, mushroom bodies and antennal lobes were dissected rapidly under binocular microscope and stored on ice in TRIzol[®] Reagent.

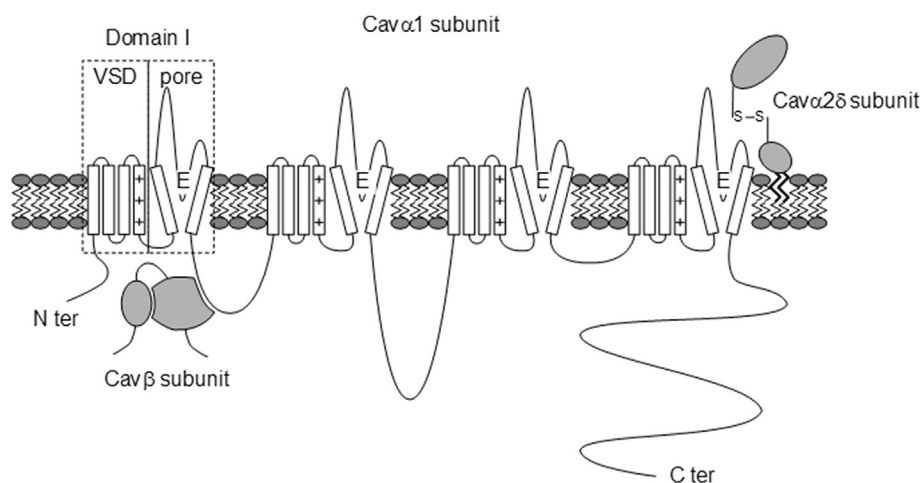


Fig. 1. Schematic representation of a voltage-gated Ca^{2+} channel. The $\text{Cav}\alpha 1$ subunit is composed of four domains (Domain I to IV) each containing six trans-membrane segments (S1–S6) and joined by linkers (the loops I–II, II–III and III–IV). The N- and C-terminus are cytoplasmic. Each domain can be divided in two functional units: the Voltage Sensor Domain (VSD), which includes the Arginine/Lysine-rich S4 segment (marked “+”), and the pore domain, which includes the P-loop with the selectivity filter (marked “E”). Cav1 and Cav2 subunits (not Cav3) are associated with auxiliary subunits: the cytoplasmic $\text{Cav}\beta$ subunit, which interacts with $\text{Cav}\alpha$ on the I–II loop, and the membrane-anchored $\text{Cav}\alpha 2\delta$ subunit.

2.2. Molecular biology

Total RNA was isolated from various tissues using TRIzol[®] Reagent according to the manufacturer's instructions. First-strand cDNA was obtained by reverse transcription of total RNA (1 µg from mushroom bodies or antennal lobes and 3 µg for the other tissues) using Oligo-dT primers and the Superscript II Reverse Transcriptase (Life Technologies) according to the manufacturer's instructions. PCR reactions were carried out with Herculase II Fusion Polymerase (Agilent Technologies). The 5' end region of the different cDNAs was obtained by Rapid Amplification of cDNA Ends (RACE) PCR using the GeneRacer kit (Life Technologies). PCR fragments were gel-purified and cloned in the pBluescript-SK vector (Agilent technologies) for sequence analysis. Full length cDNAs were obtained by sequential ligation of the different PCR fragments using endogenous restriction sites. Fragments covering the entire Open Reading Frame (ORF) were finally cloned into the pcDNA3.1 (+) vector (Life Technologies). The Alfalfa Mosaic Virus (AMV) sequence was added immediately before the start codon and the 3'-UTR sequence of the *Xenopus* β-globin gene after the stop codon to boost expression in *Xenopus* oocytes (Venkatachalan et al., 2007). Vectors were linearized and cRNAs were *in vitro* transcribed using the T7 mMESSAGEMACHINE kit (Life Technologies). Expression of the AmelCavα and AmelCavα2δ subunits during honeybee development and in various tissues from adult honeybees was assessed by RT-PCR analysis with the following primers: amcav1-031S (5'-GATTGGGCAAGTACTGCGATCCG-3') and amcav1-012AS (5'-GAACACTAGAATCTATCGGCCGATGG-3') to amplify nucleotides 5587-6015 of AmelCav1a (C-terminus); amcav2-003S (5'-GCTGCTCAGCTCGATGCGTAGC-3') and amcav2-011AS (5'-CCTCCTCTTCCTCGTTCTCGG-3') to amplify nucleotides 1767-2149 of AmelCav2a (II-S5 and linker II-III); amcav3-006S (5'-CAACATTGAACCTTGACGACTC-3') and amcav3-013AS (5'-AAGAGAATTGGCCGAGAGCA-3') to amplify nucleotides 6828-7512 of AmelCav3a (C-terminus); primers amcava2d-027S (5'-TAGTCTACCAGAACATGACCACACCG-3') and amcava2d-015AS (5'-CAAACACTGGGTTACCTTCGCA-3') to amplify nucleotides 2068-2474 of AmelCavα2δ1 (3' end of the α2 peptide); amcava2d-030S (5'-TTCCGACAAAACACTACGAGTCAGATCC-3') and amcava2d-021AS (5'-TTGCAATAGTTTTTCCCATGCCTG-3') to amplify nucleotides 711-1035 of AmelCavα2δ2 (5' end of the α2 peptide); and amcava2d-006S (5'-ATCCTCGACACGTTAGGCCCG-3') and amcava2d-002AS (5'-TGATGTAAATGGGCCGTCGTATTC-3') to amplify nucleotides 1066-1938 of AmelCavα2δ3 (VWA and Cache domains). Sequence alignment and amino acid sequence homologies between the honeybee and human subunits were determined with the Vector NTI program (Life Technologies).

2.3. *Xenopus* oocytes preparation and injection

Preparation of *Xenopus* oocytes was previously described (Cens et al., 1996). Mixtures of different cRNAs (1 µg/µl for each AmelCavα ± AmelCavβ ± AmelCavα2δ; mol:mol:mol) were injected as previously described (Cens et al., 1996). About 30 *Xenopus* oocytes were injected with 1 µl of solution.

2.4. Electrophysiological recordings and current analysis

Ba²⁺ currents were recorded as previously described (Cens et al., 2013). Current–voltage curves were fitted using the following equation:

$$I/I_{max} = G*(V - E_{rev})/(1 - \exp((V - V_a)/k_a))$$

where I is the current amplitude measured during depolarization V (from -80 to $+50$ mV); I_{max} is the peak current amplitude measured at the maximum of the current–voltage curve; G is the normalized macroscopic conductance, E_{rev} is the apparent extrapolated reversal potential, V_a is the potential for half activation, and k_a is a slope factor.

3. Results and discussion

3.1. Molecular cloning of three honey bee Cavα subunits

To clone the cDNAs encoding the three honeybee Cavα subunits, we employed conventional RT-PCR strategy using total RNA from whole adult heads and primers based on the predicted cDNA sequences (Amel_1.0, Weinstock et al., 2006) to amplify the coding sequences up to the 3' end. To obtain the 5' ends we used a RACE protocol. Despite numerous attempts, we could not amplify the complete Open Reading Frame (ORF) of each Cavα subunit in a single PCR reaction, and we produced the complete cDNAs by ligation of overlapping PCR fragments. This strategy allowed us to identify several splice variants in the sequence of AmelCav2 and two mutually exclusive exons in the sequence of AmelCav3, but none in AmelCav1. However, future experiments may reveal also AmelCav1 variants and additional variants for the AmelCav2 and AmelCav3 subunits as suggested by the last release (Amel_4.5) of the *A. mellifera* genome assembly (Elsik et al., 2014).

3.1.1. The Cav1 subunit

The AmelCav1a genomic sequence contains 41 exons that are predicted to span 29 kb (Supplementary Fig. 1). The AmelCav1a cDNA (deposited in Genbank: KJ485704¹) contains an ORF of 5934 bp encoding a protein of 1978 amino acids (Fig. 2). We found that exons 1 and 21 contained nucleotides that had not been identified in the genome assembly (neither the Amel1.0 nor the Amel4.5 release). This partially explains the differences between the AmelCav1a and the predicted Cav1 sequence (Genbank: XM_003251258.1 that has been removed and replaced by a new version XM_006569121.1 since the beginning of this study). The sequence of exon 1, which we obtained by RACE, is missing in the latest prediction of the Cav1 sequence. However, the deduced amino acid sequence of AmelCav1a N-terminus is very similar to that of the predicted Cav1 subunits in other arthropod species, for examples *Bombus terrestris* or *Harpegnathos saltator* (Genbank: XP_003393703 and EFN84355, respectively, not shown). Exon 21 encodes the trans-membrane segments S2 and S3 of domain III (IIIS2 and IIIS3, see Fig. 1) of the Cavα subunit. The AmelCav1a C-terminus contains a stretch of glutamine reminiscent of a polyglutamine tract (polyQ, Fig. 2). In mammals, polyQ sequences are found in many proteins (Butland et al., 2007) and in humans, polyQ motifs with a number of glutamines that exceeds a certain threshold have been implicated in severe neurodegenerative diseases (Gatchel and Zoghbi, 2005; Williams and Paulson, 2008). Spinocerebellar Ataxia Type 6 is caused by an abnormally long (>20 glutamines) polyQ tract in the C-terminus of the Cav2.1 Ca²⁺ channel subunit. To determine whether in honeybees, like in humans, the polyQ length in AmelCav1a is variable, we collected

¹ The sequences deposited in the Genbank under the number KJ485704–KJ485709 and JX997993 are patented (patent # FR 3003863/WO 2014155021).

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Ame1Cav1a (1) -----MSAGGDGSGSLG PPELAGAQPT AATPP ILGQHRNPET GQDGGQAATAQSQTGQTLGT STGAA
Ame1Cav2a (1) -----
Ame1Cav3a (1) MSLHY PQGYR YRPAS KAVGS GNGEQGSDSDVAELS DLEDDDEDEGGQSTNRCVE QNVKEEKEDEDDNE DEDDD

(63) AAAAAAAAAAKSATKRPARGGKAPP----- DRPNRTLFLCL PLKNPLRKMCI DVVVEWKPFEWLIIMTI FANCI
(1) ----MAATS PCLPLEVGSARGG----- KGTTS LFILSEDNCRKHXTRFI IEWPPFEYAVLLT I IANCV
(76) DNE DDEDQEDEEE DENEE DNGVDVDGDEL PYPGFVVALRYLDQNTNPRNWC LALITNPFWERVSMVILLNCI
IS1

(131) ALAVYTPYPYGDSNLTNQYLEKIEYIFLVI FTVECVMKI IAYGFVAH PGAYLRNGWN ILDFS IVVIGMVSTVLSV
(60) VLAL EEHL PKQDKT I LAQKLEAT E IYFLGIFCVEASL KILALG FVLHRGSYLRNIWNI MDFVVTGFI TAFS QG
(151) ILGMYQFCVDDQCVI NRCKI LQMFDDI IFA FFSLEMT IKMVAMGI YGKGYLADSNRRLDFE IVIAGALEYCLNV
IS2 IS3

(206) LMKEGFDVKALRAFVRLRPLRLVSGVPSLQVVLNSILRAMI PLLHIALLVLFVII YAIIGLELFGSKMHKT CRH
(135) IELD-MDLRTLRAIRVLRPLKLVSGIPSLQVVLKSI I KAMAPLLQIGLLVFAIIVFAI IGLEFYSGTLHKT CYS
(226) ENMN---LSAIRTIRVLRPLRAINRIPSMRILVMLLLDTPMLGNVLLLCFFVFFIFGIVGVQLWEGILRQRCFL
IS4 IS5

(281) NMTDAIMDDP-----VPCGPGG-----YQCDNVGSDYY
(209) IRDINVI VKEGE Q-----ASPCNTDNKSE-----APFGAHVCDANIS
(298) KALPNVKY PDDLEKYFEYQGDYICSRPDDNGMHSCSNLPLKLGNVVCCNNTALPNNTTFI TNDTCVNWNY YTT

(309) CSKQFWE GPNWG ITNFDNFGLAMLT VFCVTL EGWTEVLYNIEDAMGSSWQNIYF ISMVI LGAFFVMNLI LGVLS
(246) TCMDHWE GPNFG ITSFDNIGFAMLT VFCI IMEGWTA ILYWINDALGSTYNNIYF I PLIVLGSFFMLNLV LGVLS
(373) ECKGQGNPFGTISFDNIGLAWVAIFLVISLEGTWIDIMYVQDAH-SFWDNIYFVLLIVIGSFFMINLCLVVIA
* # IS6

(384) GEFSKERE-----KAKARGDF
(321) GEFAKERE-----KVENRQSF
(447) TQFSETKKREMERMLERARFHSTSTLASSNTI SEPTTCYAEIVKYIAHLWRGKRRLMKRYRVVLYKRQQKREQ

(400) HKLREKQ-----
(337) LKLRRQQ-----
(522) NLLKEQQQHGHFRGGA PNSDSNRLPGDRRLHGRCPRLLAAL EYAEQQQQQQGIGSGGSLTDFPANSPTQT

(407) -----
(344) -----
(597) AILATAIGNIGG SGNLAIAPRASPEVSEADVSLNVYNRIGLHRTSSVSCNGSDNVL SNATEASIQTNVLLSPP

(407) -----
(344) -----
(672) CTHYRRRSSVMFSDVLLHGSNNIGNTLQAGMAVSPGERNVCSSEKMTQTGDGNVWS SPLPDHIQMQAELGGNEA

(407) -----QIEDDLRGYLDWITQAEEDIE PETDEPKMQDGKTKQQ-----SEMESTDQLE GDEEGVQQ
(344) -----QLEHELYCYLNWICKAEVILAEERTTEEE KKHILEGRKRAEAKKKGKSKSTDTEEEE GDDQDDG
(747) MTCQELLALS GALS AALPTGQLALDSFLNSFTKGITDRHITLEDRTQWLASDIDNCSCCCELQGIDQWPDEGDKW

(461) ESLWRRKK-----LDFDRVNRMRRA CRK#AVKSQVFWLIIVLVFLNTGVLATEHYNQPHWLD DDFQEITNMF FI
(412) FSRSSSTKEGFC-KQFWLAEKRFRYWRKSVKSQKFYWFVIVLVFFNTVCVAVEHYGQPQWLTDFLYFAEFVFL
(822) TKNSRAKRFLRSCGNSCICAIR CIRRLIKKLVEHKYFOQGILLAILINTLSMGI EYHNQPEQLTIVVEVSNIVFS
IIS1 IIS2

(530) ALFTMEMMLKMYSLGFQGYFVSLFNRFDCFVIGSITEMILTNTHVMPPLGVSVLR CVRLLRVFKVTKYWRSL
(486) ALFMLEMFIKVIYALGPRTYFDSFNRFDCVVI SGSI FEVIWSEVKSGS--FGLSVLRALRLRI FKVTKYWRSLR
(897) AVFAVEMLLKIIAEGPFGYISNGFNVDGVVVLSVVEICQAFVEERGGSSGLSVLRTFRLLRILKLVRF LFNLR
IIS3 IIS4

(604) NLVASLLNSIQS IASLLLLFL FIVIFALLGMQVFGGKFNFNLEN-----KPRHN FDSFWQSL
(559) NLVISLLSSMRS IISLLFLLFL FILIFALLGMQLFGGQFNFD SG-----TPPTNFNTFP IAL
(972) RQLFVMLRTMDNVAVFSLLVLFIFIFS ILGMYLFGGKFCMWA DRSRPTCAEVVSRHPLCRC DRKHFNDIVWAL
IIS5

(663) LTVFQILTGEDWNAVMYDGIRAYGGVSGFMLACFYI IILFI CGNYI LLNVFLAI AVDNLANADAESLTAIEKEAEE
(616) LTVFQILTGEDWNEVMYQGIESQG-GHKKGM IYSLYFIVLVLF GNNTLLNVFLAI AVDNLANAQELSAAENE EEE
(1047) VTVFQILITQEDWNVVLFNGMQKTS-----HWAALYFVALMTFGN YVLFNLLVAI LVEGFSSERNERREREQREM
* # IIS6

(738) EAEKNKSHSASPTRDKD-----SGEQDGGEGT GGEDEGGGIDLEHDPNETME DYE
(690) EDKQKQ-----AQEIEKEIQSLQNPKG GAGPKVEICP PSPNQNFK
(1116) ARLAAKETGIGSDDGS SRISRSHSIT DSDTYTQDRKNSWQS AEELHKYKDNNSKEKQNTVWKHQIDE PKCN IQKV

(790) AAVDTIETSEK-----
(730) DGKGGKQS-----
(1191) NKMKGSTGQPP IITHATAATPQDSPNT ILDVGRVYPTAALS IESIDRSGSQCSISS GLLKLPDVS NKIPTKNLIA

(800) -----SDDMNT HAKVRLNI ESDEEVEEEE EVEHNEMHGERFIYD GTE
(738) -----SEEEKKQ-----
(1266) GQFPRRINLVTAVVNP TMRES SNSSS PRIQRGYSWKLSRPS LRKKRWLQTE DESPRATV LNNGRS TILGSNSTF

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Fig. 2. Alignment of the AmelCav α subunit amino acid sequences. Dashes represent gaps introduced to maximize sequence alignment. The six trans-membrane segments (S1–S6) of each domain (I to IV) are indicated under the sequences. The amino acids that define the AmelCav1 α and AmelCav2 α Alpha Interacting Domain (AID) involved in Cav α –Cav β interaction are boxed. The EEEE and DCS loci, important for selectivity and conductance, are marked by * and #, respectively, in each domain. The polyQ tract in the AmelCav1 α C-terminus is highlighted in bold. Sequences were deposited in Genbank under accession numbers KJ485704–KJ485706.

Ame1Cav1a (842) QGVSARPRRMSEFNMATKKQ-----
 Ame1Cav2a (745) -----DEDDDTGPK-----
 Ame1Cav3a (1341) NGGYLHGSIRNDTQSDTPNNRRTVLTSSNRSLSPPNNSIESRSSSIRRYTATPNQMRWISDLSRNSLRENENVQS

 (862) -----
 (754) -----
 (1416) PTRKTLPLDEVPMQCSTARTINNLSMETGPLPRIKRLPDQDDNPRTDEQT PPLNGHGSASSIERIKKIFMFFEP

 (862) ----PIPAGSAFFIFSQTNRIRIFCHWLCNHSTFGNVILVCIMISSAMLAAEDP-LRASSSRNLVLQKFDYF FTT
 (754) ----PMLPYSSMFILSPINPVRRAAHWVNLRYDFDFIMVVI SLSSI ALAAEDP-VWEDSPRNEVLNYFDYA FTG
 (1491) KGCLKERDDYSLYIFPNNRFRVLCRLLDVQRWFDNVVLF FIGLNCITLAMERPNI PPD SGERL FLSTANYIFTG

 III S1 III S2
 (932) VFTIEICLKMSYGFII HEGAF CRSAFNLLDLLVCS SLISMSFSSG-----AFSVVKVLRVLRVLRPLRA INR
 (824) VFTVEMILKIIDLGIILHGPSYLRFEWNIMDAVVVICAAVSFAFDMT GSSAGQNLST IKSRLVLRVLRPLKTIKR
 (1566) VFAVEMFIKVVASGMLYGSDAYFTS GWNIMDVGVLV I I I I DLSMSLLSSSSPRIFGLIRVRLRLSLRPLRVINR
 III S3 III S4
 (1001) AKGLKHVVQCVIVAVKTI GNIIVLVTISLLQFVFAVVGVLQFKGKFFYCTDASKMTKEECQGT YLEFENGNINKPIM
 (899) VPKLKAVFDCVVNSLKNVINILIVYILFQFI FAVIAVQLFNGKFFYC SDESKYTQDCQGGY FVFEDGALLPEPK
 (1641) APGLKLVVQTLSSLRPIGNIVLICTF FVIFGILGVLQFKGAFYYCE GPDIKNVRNKTDCLADKRN---VWLN R
 III S5
 (1076) KERNWCQQRHFDDVAKAMLT LFTVSTFE GWPSLLDYSIDSNKEDHGPIHNFRIVAAYYI IYII I IAFFMVNIF
 (974) K-REWQS QFFHYDNVMAAMLTL FAVQT GEGWPKILQNSMAATYEDKGPION FRIEMSI FYI VYFIV FFFVNI F
 (1713) K-----YNFDDL GKALMSLFVLS SRD GWNIMY TGLDA VGVDDQPIENYSE WRLLYFIA FILLVGGFFVLMNF
 * # III S6
 (1151) VGFVIVTFQNEGEQYKNC ELDKNQRNCIEFALKAKPVRRYIPKHR---IQYKVVWFVTSQPF EYTI FTLMINT
 (1048) VALIIITFQEQGEAELQDGEIDKNQKSCIDFTIQAR PLERYMPKERN-SVKYIWRIVVST PFEYFIMGLIVLNT
 (1780) VGVVVENFHRCREEQE KEERVRAAKRALQMEKKRRKMHEP PYYTNYKSRLFVHNVVTSKYFDLATAAVIGLNV
 IVS1
 (1223) VTLAMKFYRQPEIYITQALDVLNMI FTAVFALEFIKLA AFRFKNYFGDAWNVDFDI IVLGSFIDIVYSEVNP---
 (1122) VLLMMKFHRQS DAYKNTLKYMNMCFT GMFTVECILK IAAFVGRNFFKDAWNTDFDI TVIGSIVDALVIEFG----
 (1855) VTMAMEFYMPKALTYALKI FNYFFTAFILES FMKLLALGLHLYLKDKNQLDVGIVILSVVGVILEEVESKI I
 IVS2 IVS3
 (1295) GSTIISINFFRLFRVRLVVKLSRGE GIRTLLWTFIKSFQALPYVALLIIMLFFIYAVIGMQVFGKIAIDDETSI
 (1193) -ENFINVFLRLFRARLIKLRQGYTIRILLWTFVQSFKALPYVCLLIAMLFFIYAIIGMQVFGNIALDADTSI
 (1930) P INPTI IRVMRVLRIARVLKLLKMAKGI RALLDTVMQALPQVGNLGLLFFLLFFIFAALGVELFGRLECSDDMPC
 IVS4 IVS5
 (1370) N---RNNNFQSFPQAVLVLFRSATGESWQEBIMMDCSVQPGKVKCDPNS DEALNTNGCGSDIAFPYFISFYVLC SF
 (1267) T---KHNNFQSFIQGLMLLFRCATGEAWPNIMLSCVKGR-----PCDAKAGKQEGGCGSNIAAYFVSVFIFFC SF
 (2005) QGLGEHAHFSNFGMAFLTLFRVATGDNWNGIMKDTLRDD-----CDEAADCVKNCCVSTII IAPIFFVIVLMAQF
 * # IVS6
 (1442) LIINL FVAVIMDNFDYLTRDWSILGPHHLDE FIRLWSEYDPAKGR IKHLDVVTLLRKISPLGFGKLCPHRVAC
 (1334) LMLNLFVAVIMDNFDYLTRDSSILGAHHLDE FVRIWAEYDPNATGKIHYTEYDMLKNMDFPLGFGNKCPNRLAY
 (2075) VLVNVVAVLMLKHL EESHKQMEDELDMETQLERE LAAEQEELLEVEDEE DDETIKRERDDGDI DEDDEDVREHS
 (1517) KRLVSMNMPNLDGTVLFNATLFAVVRTSLR IKTEGN--IDDANAE LRAVIKKIWKRTSPKLLDQVVPVPPG--GD
 (1409) KKLIRMNMPVDVLDKVNFTTTLFALI RENLN IKVRRASERNQANEE LRDTIRSIWPLQAKKMLDLLIPRNEEIGR
 (2150) ILVANEKIPASRPGLAKVRS L PANFIYNPPRERNAD DGTISVSLARRSSYHRSSSRPSKFKSKRRQTFHSHGHQR
 (1588) DEVTVGKFIYATFLIQDYFRFRKRRKEQEMKGDKECHNTVT LQAGLRTLHEAGPELKRAISGNLEE LLDNPEPM
 (1484) GKMTVIGKIYVCLLILESWRTTRFGQIESAGQNDNDLIDNDNAGQ-----SPAAQAMELQDVVV
 (2225) RSLLEPMHFEVAEVFEKPVSNLSVPIIPQHNYDATSRDITHVQR-----QRLQATSDGQENK
 (1663) HRRNHS LFGSVWSSMRKGHSFNRRARSLKVNSTSKASPTNSIDFVPYSSFHRRGGDPSNQI TARSHQVVPNVAGG
 (1542) SDSRAGSLES LTHGKRLHPPVQVVRHPSRS PSLRRHSPGRPGYDHGHYYHEG-----PG
 (2282) SPLNVSKPPSSSSPMSLAGSVTTLT CPKISERYLMPTFN IYPSKATLSCRPS-----
 (1738) LSDSAMNQMGIDPKLTGIEESIPLRPLAVFGNFPVQQSYHHTSYKVLDPGPGSGNYLHPNNE YVSWAGESNGSIGA
 (1598) FSDIVSNVVEIQRHTHHPHPSQYNHRHRMRDYDCHDYDDGPWSASTSPARTPSP IHHIDRGRHYGTTSLERQS
 (2336) -----SEAMQS QFDTNVSI GSVSKT DSTMNGYMETGVPRS NGDRS NDSKEKNERRVSA PPTDDLVDQSIINERR
 (1813) ERLSHSLPGSPADRKNFEVI GSAESLVGRVLEQGLGKYC DPDFVRYTSREMQEALDMTREEMDRAAHQQLLQE
 (1673) RSPSPIGGRQP PHTHQYHRHHPHQHSYPVLVTRRGGRRLPPTPNKPS TLQLK PANINFPKLNASPTQHS HIH-
 (2406) P SKLKS GNLTETMRIVSDQSSSTRIE SYGQVYVTEERFEEVSPMSTGNMSDSIIGS SGTNGSESVT GSGTGSAS-
 (1888) RRGQPLSYQLQGVDDQWTSYYSQPSQSTGIGYQPLQEQQSSGQPRQYRSYYRGGGQATTISDPSSI QQQQQQQQQ
 (1747) ---VPI PAMQHPPPGQHLPPMQPS-----HCPLSEFQAVAMGR-----GGRLLPSPVPNGYKQPQAKQR
 (2480) -----GS-GSRNESAVMHVTEGVCATIGSDVRIYVD DTDSSANNHQRKANEGTPEVSMI ISSVIAIPDVTIEE
 (1963) QQQQQQQQQQQQSPPS--
 (1805) TPRSRLSDSDEDDWC---
 (2549) ERSERFDVPSSDGPSPDPS

Fig. 2. (continued).

Table 1
Analysis of AmelCav1 C-terminal sequence in DNA isolated from honeybees collected in different countries.

Country	City	Sequence	Number of Gln	n
France	Rochefort	GGQ A TTTSDP S SIQQQQQQQQQQQQQQQQQQQ----SPPS	21	2
	QQQQQQQQQQQQQQQQQQQ----....	22	
	St Mathieu de TréviérsQQQQQQQQQQQQQQQQQQQ-----....	18	4
	QQQQQQQQQQQQQQQQQQQ----....	21	
	QQQQQQQQQQQQQQQQQQQ-----....	18	
	QQQQQQQQQQQQQQQQQQQ----....	22	
	AvignonQQQQQQQQQQQQQQQQQQQ-----....	21	1
	QQQQQQQQQQQQQQQQQQQ-----....	21	
	QQQQQQQQQQQQQQQQQQQ-----....	25	
	QQQQQQQQQQQQQQQQQQQ-----....	22	
	QQQQQQQQQQQQQQQQQQQQ-----....	25	
	QQQQQQQQQQQQQQQQQQQ-----....	19	
... T TQQQQQQQQQQQQQQQQQQQ-----....		25		
.....QQQQQQQQQQQQQQQQQQQ-----....		25		
Turkey	Eskischir	... T TQQQQQQQQQQQQQQQQQQQ-----....	19	2
	QQQQQQQQQQQQQQQQQQQ-----....	21	
Algeria	BoufarikQQQQQQQQQQQQQQQQQQQ-----....	21	1
	QQQQQQQQQQQQQQQQQQQ-----....	22	
	QQQQQQQQQQQQQQQQQQQ-----....	22	
Bulgaria	BirimirziQQQQQQQQQQQQQQQQQQQ-----....	21	2
	QQQQQQQQQQQQQQQQQQQ-----....	21	

Dots represent identical amino acids and dashes gaps in the alignment.

specimens (n = 18) from different locations in France, Algeria, Turkey and Bulgaria (Table 1). We found that they were mostly (14/18) heterozygous and that the number of glutamine residues in the polyQ motif varied between 18 and 25. Although this seems very limited compared to humans, where it may range from 7 to 16 in healthy individuals (Butland et al., 2007), we cannot exclude that by increasing the sample size we may detect a larger polyQ length variability also in honeybees. A BLAST analysis using the AmelCav1a C-terminus showed that the polyQ tract was absent in the otherwise very similar *B. terrestris* and *Bombus impatiens* Cav1 sequences (Genbank: XP_003393703 and XP_003490120, respectively), but present in the *Apis florea* and *Apis dorsata* Cav1 sequences (Genbank: XP_003697046 and XP_006618300, respectively). This suggests that the presence of a polyQ tract in Cav1 is specific to the *Apis* genus. Moreover, it is quite surprising to find a polyQ of “pathological” length (up to 25 glutamine residues) in the C-terminus of honeybee Cav1, but this could help understanding the consequences of its presence in human Ca²⁺ channels.

3.1.2. The Cav2 subunit

Cav2 genomic sequence contains 34 exons, the shortest (exon 29) leading to the addition of only three amino acids, that are predicted to span 77 kb (Supplementary Fig. 1). The AmelCav2a cDNA (deposited in Genbank: KJ485705) contains an ORF of 5460 bp encoding a protein of 1819 amino acids (Fig. 2). The predicted cDNA for the Cav2 subunit available at the beginning of this work (Genbank: XM_392298) lacked exons 27 and 32, and differed from AmelCav2a at the N-terminus and at the junctions

between exons 10 and 11, 13 and 14 and 33 and 34. The differences observed at the junctions were not present in the new assembly of the honeybee genome (Amel_4.5) that also confirmed the presence of the exons 27 and 32. The single remaining difference concerns the AmelCav2a N-terminus which is not found in any of the predicted Cav2 isoforms. Moreover, we could not amplify, using specific primers, the long 5' end of Cav2 that is present in the outdated (Genbank: XM_392298) and also in the new version (Genbank: XM_006557941) of the predicted Cav2 sequence. We identified by RACE another N-terminus resulting from the alternative joining of exons 2 and 3 (not shown). This alternative N-terminus is present in some isoforms of the predicted Cav2 (Genbank: XM_006557953 and XM_006557954). Exons 20, 27, 28, 29 and 32 are alternatively spliced, whereas exon 23a and 23b are mutually exclusive (Supplementary Fig. 1). The insertion of exon 20 adds 16 amino acids to the S3–S4 linker of Domain IV (Fig. 3A). Interestingly, the insertion of an alternative exon encoding only two amino acids at a similar position in the mammalian Cav2.1 subunit strikingly modifies the channel sensitivity to inhibition by the toxin ω -Agariva (Bourinet et al., 1999; Hans et al., 1999). It is, therefore, tempting to speculate that the insertion of exon 20 might modify the pharmacology of the honeybee Cav2 channel. This additional sequence may also affect the sensitivity of the channel to transmembrane voltage and/or to external ions. Indeed, specific amino acids similarly located in the S3–S4 linker of the mammalian Cav3.2 and Cav2.3 subunits are involved in the channel sensitivity to extracellular Ni²⁺ or H⁺, respectively (Cens et al., 2011; Kang et al., 2006). Exon 23 encodes the beginning of

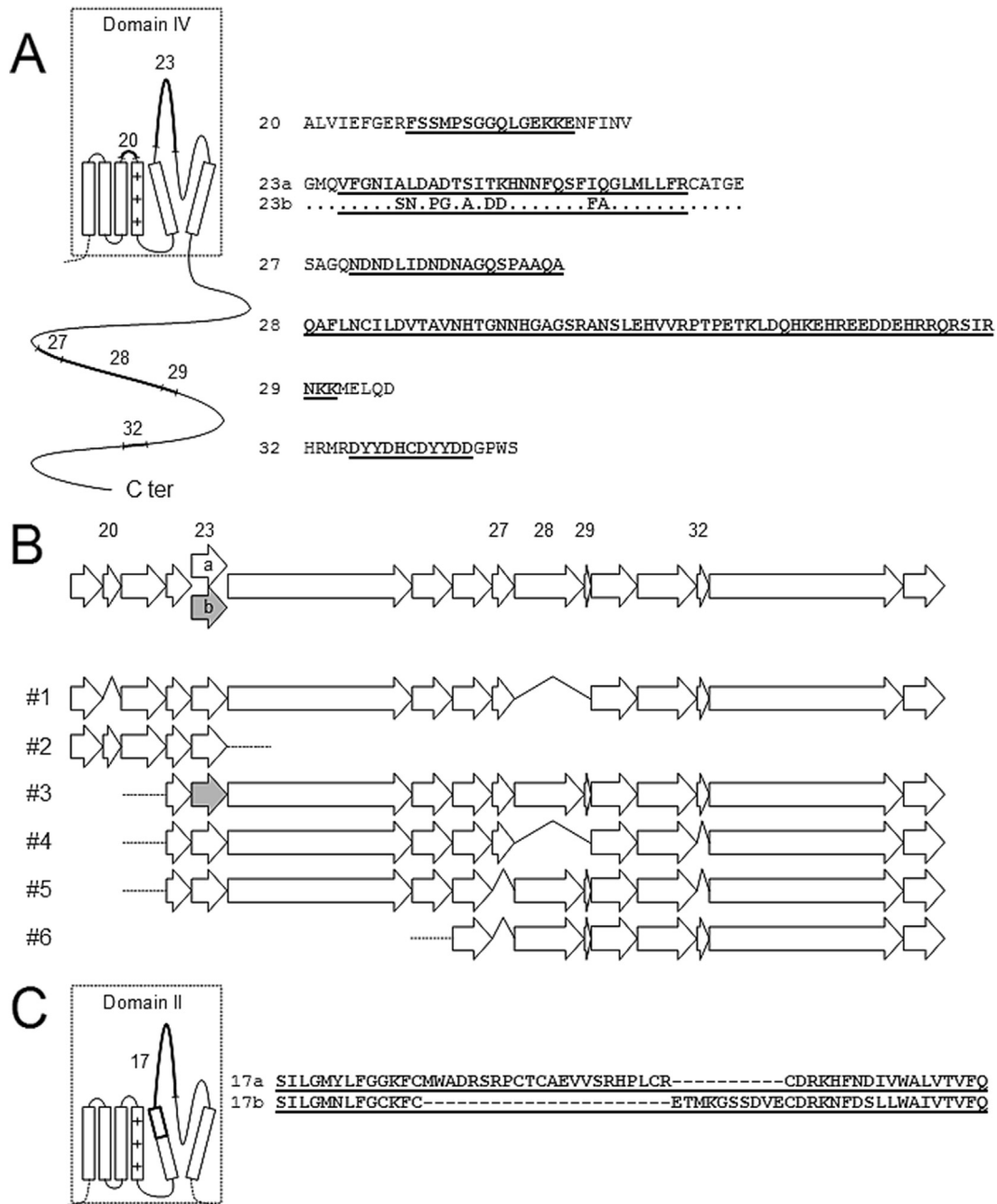


Fig. 3. Alternative and mutually exclusive exons of the AmelCav2 and AmelCav3 subunits. **A.** Schematic representation of Domain IV of the AmelCav2 subunit and location of the alternative exons 20, 27, 28, 29 and 32 and of the mutually exclusive exons 23a and 23b with their respective amino acid sequences (bold and underlined). Dots represent similar amino acids. **B.** Combination of coding exons in six independent PCR fragments of AmelCav2 (#1 to #6; sequences were deposited in Genbank under the accession numbers KM577626%5fKM577631). **C.** Schematic representation of AmelCav3 Domain II and location of the mutually exclusive exons 17a and 17b with their respective amino acid sequences. Dashes represent gaps in the amino acid sequences. The sequence of the fragment including exon 17b has been deposited in Genbank (accession number KM577632).

the S5–S6 linker of Domain IV (Fig. 3A). Exon 23a differs from exon 23b by only nine amino acids, but this difference could influence the channel conductance because these residues are located in the outer mouth of the channel pore (Cens et al., 2011). Exons 27, 28, 29 and 32 are located in the C-terminus (Fig. 3A) that, in mammalian Cav1 and Cav2, contains several domains that can modify the channel biophysical properties, such as Ca^{2+} -dependent facilitation and Ca^{2+} -dependent inactivation (Cens et al., 2006) or the regulation of these properties by Ca^{2+}

binding proteins (Lee et al., 2002). With the exception of exons 28 and 29, which were always found together, the exons of AmelCav2 C-terminus seem to be expressed in multiple combinations (Fig. 3B). This considerably increases the number of Cav α subunits that may harbor specific properties.

3.1.3. The Cav3 subunit

The genomic sequence of AmelCav3 contains 36 exons that are predicted to span 190 kb (Supplementary Fig. 1). Exons 17a

and 17b are mutually exclusive. AmelCav3a cDNA (Genbank: KJ485706) contains an ORF of 7698 bp encoding a protein of 2566 amino acids (Fig. 2). Probably because of the poor quality of the genomic sequence, there was no predicted full length Cav3 subunit in the honeybee genome, when we started this study, but only a partial sequence (Genbank: XM_624269). Indeed, exons 5, 10, 19, 22 and 26 of AmelCav3 contain nucleotides that were not identified in the first assembly of the honeybee genome (Amel_1.0). Nevertheless, AmelCav3a sequence is close to the predicted Cav3 sequence in other arthropod species, such as *A. florea* (Genbank: XP_003698162). The last assembly of the honeybee genome (Amel_4.5) contains a predicted cDNA for the Cav3 subunit (Genbank: XM_006562038) that is very close to that of AmelCav3a. Exons 17a and 17b encode a 53 or 41 amino acid-long fragment in the S5–S6 linker of Domain II (Fig. 3C). As described for exon 21 of AmelCav2, this exchange could influence the conductance of the honeybee Cav3 channel. Moreover, like in mammals, where it plays a critical role in Cav3 channel trafficking and gating (Vitko et al., 2007), the I-II linker is much longer in AmelCav3a than in the other two Cav α subunits (Fig. 2). It also contains a glutamine stretch that is shorter than the polyQ tract found in AmelCav1a C-terminus. However, we found an extra glutamine in a PCR fragment encompassing the same sequence, suggesting that this “polyQ” motif, like that of AmelCav1, is subject to length variability (not shown).

3.2. Comparison of the honeybee and the human Cav α subunits

The degree of homology between honeybee Cav α subunits was comparable to that found between human Cav α subunits

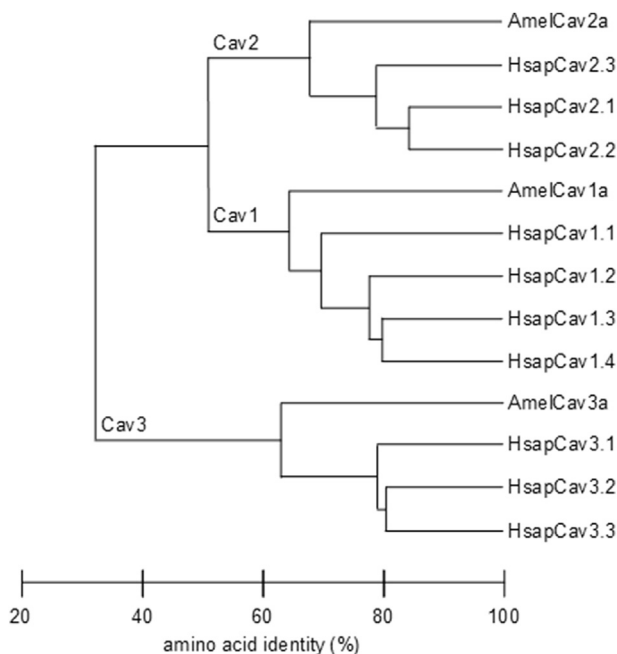


Fig. 4. Dendrogram illustrating the similarity of the honeybee and human Cav α subunits. The dendrogram was drawn using the Phylogeny platform (Dereeper et al., 2008) (www.phylogeny.fr) and MUSCLE3.6 and PhyML3.0 and the following human sequences: Cav1.1 (AAA51902), Cav1.2 (AAA51900), Cav1.3 (NP_000711), Cav1.4 (CAA12175), Cav2.1 (AAB64179), Cav2.2 (AAA51897), Cav2.3 (AAA72125), Cav3.1 (AAD29401), Cav3.2 (NP_066921) and Cav3.3 (AAF44626). Only the sequences of the domains I to IV were used for this analysis.

(Supplementary Table 1). Moreover, when we specifically compared the three Cav1, Cav2 or Cav3 families, none of the honeybee Cav α subunits showed a particularly high homology with a specific human subunit (Supplementary Table 1). As expected, the homology between honeybee and human Cav α subunits was particularly important within the Domains (Fig. 4) whereas the connecting linkers and the N- and the C-terminus differed substantially. However, the AmelCav1a proximal C-terminus, which contains the pre-IQ and IQ domains that are involved in the interaction with the Ca²⁺ binding protein calmodulin and are therefore important for the Ca²⁺-dependent regulation of channel properties (Cens et al., 2006), presented a strong homology with its mammalian counterpart, like in other invertebrates (Tsiakina et al., 2013). This suggests that such regulation may also exist in honeybee Ca²⁺ channels.

3.2.1. The S4 segments

Fig. 5 displays an alignment of the S4 segments of each Domain (IS4 to IVS4) of the honeybee and human Cav α subunits. The presence of regularly spaced basic amino acids (arginine or lysine) allows the S4 segments to play the role of voltage-sensors that move through the membrane in response to modifications of the membrane potential. Various mechanisms (reviewed in Tombola et al., 2006; Bezanilla, 2008) have been proposed to explain the coupling between S4 movement and channel opening. Although it would be highly speculative to favor one mechanism rather than another, it is worth noting the high conservation of the S4 segment sequences in the honeybee and human Cav α subunits. This suggests a relative similarity of the voltage-dependent properties of these channels in agreement with Ca²⁺ current recordings realized in honeybee neurons and muscle cells (Collet, 2009; Kloppenburg et al., 1999; Laurent et al., 2002; Schäfer et al., 1994). However, the lack of specific blockers/markers did not allow concluding whether the recorded currents resulted from the activity of a single or several types of Ca²⁺ channels. The presence of LVA Ca²⁺ channels in honeybee tissues has not been unambiguously reported yet. However, the hump in some current–voltage curves that appeared at low voltage suggests the existence of such Ca²⁺ channels in honeybee muscle cells (Cens et al., 2013). It has recently been shown that the Cav2 subunit may produce both HVA and LVA Ca²⁺ currents in *Drosophila* (Ryglewski et al., 2012). In fact, only the expression of an insect Cav3 subunit, which has never been done so far, would undoubtedly prove that AmelCav3 form an LVA Ca²⁺ channel (see below).

3.2.2. The selectivity filter

The most outstanding property of voltage-gated Ca²⁺ channels is probably their remarkable specificity for Ca²⁺ ions. The ion-conducting pore is composed by the four S5, the four P-loops and the four pore-lining S6. The selectivity towards divalent cations is achieved by four negatively charged amino acids (glutamate or aspartate) that delimit the EEEE locus, a Ca²⁺-binding site located in the narrowest part of the channel pore. This locus is composed of one glutamate from each of the four P-loops of Cav1 and Cav2 subunits (EEEE). Conversely, in the Cav3 subunit, the P-loops of the first two domains contain a glutamate and the P-loops of the last two domains contain an aspartate (EEDD). Mutations at this site severely affect the Ca²⁺ channel selectivity and/or permeability (reviewed in Sather and McCleskey, 2003). The EEEE locus constitutes the molecular signature for voltage-gated Ca²⁺ channels and was perfectly conserved in the honeybee Cav α subunits as well (Fig. 6). Besides the EEEE locus, another ring of charged and non-charged amino acids, the DCS locus (for Divalent Cation Selectivity), is responsible for the specific permeation profiles of Ca²⁺ channels

		IS4					IIS4					
		+	+	+	+	+	+	+	+	+	+	
AmelCav1a	VKALRAFRVLRPLRLVSGV	231					GVSVLRCVLLRVFKVTKYW	599				
HsapCav1.1	.K..R..R..R..R.....	179					.I...R..IR..RI.K..K..	542				
HsapCav1.2	.K..R..R..R..R.....	222					.I...R..R..RI.KI.R..	605				
HsapCav1.3	.K..R..R..R..R.....	254					.I..FR..R..RI.K..RH.	653				
HsapCav1.4	.K..R..R..R..R.....	220					.I...R..R..RI.K..RH.	628				
		+	+	+	+	+	+	+	+	+	+	
AmelCav2a	LRTLRAIRVLRPLKLVSGI	159					GLSVLRALRLLRIFKVTKYW	554				
HsapCav2.1	.R..R.VR..R..K.....	209					.I...R..R..R..K..K..	597				
HsapCav2.2	.R..R.VR..R..K.....	206					.I...R..R..R..K..K..	592				
HsapCav2.3	.R..R.VR..R..K.....	204					.I...R..R..R..KI.K..	586				
		+	+	+	+	+	+	+	+	+	+	
AmelCav3a	LSAIRTIRVLRPLRAINRI	248					GLSVLRTFLLRILKLVRF	967				
HsapCav3.1	F..VR.VR..R..R...RV	194				R..R.MRV.K..R..	848				
HsapCav3.2	F..VR.VR..R..R...RV	194				R..R.MRV.K..R..	848				
HsapCav3.3	L...R.VR..R..K...RV	192				R..R..RV.K..R.M	599				
		IIIS4					IVS4					
		+	+	+	+	+	+	+	+	+	+	+
AmelCav1a	VKVLRLVLRVLRPLRAINRAK	1002					NFFRLFRVMRLVKLLSRG	1319				
HsapCav1.1	.KI.R..R..R..R...R.K	912					A..R..R..R.IK...RA	1250				
HsapCav1.2	.KI.R..R..R..R...R.K	984					T..R..R..R..K...R.	1314				
HsapCav1.3	.KI.R..R..R..R...R.K	1019					T..R..R..R..K...R.	1353				
HsapCav1.4	.KI.R..R..R..R...R.K	973					T..R..R..R..K...K.	1299				
		+	+	+	+	+	++	+	+	+	+	+
AmelCav2a	IKSLRVLRVLRPLKTIKRV	900					GFLRLFRAARLIKLLRQ	1216				
HsapCav2.1	.K..R..R..R..K..KRL.	1361					S..R..R..R..K..R..	1675				
HsapCav2.2	.K..R..R..R..K..KRL.	1266					S..R..R..R..K..R..	1582				
HsapCav2.3	.K..R..R..R..K..KRL.	1272					S..K..R..R..K..R..	1589				
		+	+	+	+	+	+	+	+	+	+	+
AmelCav3a	LRVFRLLRSLRPLRVINRAP	1642					RVMRVLRIRAVLKLKMA	1954				
HsapCav3.1	.R.LR..RT.R..R..SR.Q	1373					RI.R..R..R..K..K..	1692				
HsapCav3.2	.R.LR..RT.R..R..SR.Q	1373					RI.R..R..R..K..K..	1703				
HsapCav3.3	.R.LR..RT.R..R..SR.P	1255					RI.R..R..R..K..K..	1567				

Fig. 5. Alignment of the S4 segments of the honeybee and human Cav α subunits. The human sequences used for this alignment are the same as in Fig. 4. The charged amino acids (arginine/lysine) are marked by the plus sign. Dots represent identical amino acids.

towards divalent cations (Cens et al., 2007). Both the number of charged amino acids and their relative position in the DCS locus define the channel permeation profile. AmelCav1a DCS locus was similar to the DCS locus of mammalian Cav1.1, 1.3 and 1.4, as it had two charged amino acids and the DCS locus of AmelCav3a to that of Cav3.3. By contrast, AmelCa2a DCS locus had only one charged amino acid and thus, was clearly different to that of the three mammalian Cav2 subunits (Fig. 6).

3.3. Molecular cloning of a new variant of the honey bee Cav β subunit

We previously reported the cloning and functional expression of two variants of the honeybee Cav β subunit and the role played by their different N-terminal sequences in the specific regulation

of channel inactivation when co-expressed with a mammalian Cav α subunit (Cens et al., 2013). Mammalian genomes contain four genes that encode four distinct Cav β subunits, which are composed of two conserved domains (C1 and C2) flanked and joined by more variable regions (V1, V2 and V3, see Fig. 7A). C1 and C2 were predicted by crystallographic modeling to contain a src homology 3 (SH3) domain and a guanylate kinase-like (GK) domain, an association that characterizes the MAGUK family of scaffolding proteins (Chen et al., 2004; Hanlon et al., 1999; Opatowsky et al., 2004; Van Petegem et al., 2004). The SH3 domain is split by an intervening sequence of variable length (the HOOK domain) that connects C1 and C2 (Fig. 7A). The GK domain binds tightly to the I-II loop of the Cav α subunit through the alpha-interaction domain (AID, see Fig. 1) and is involved in intramolecular coupling with the SH3 domain (reviewed in

	Loop I		Loop II		
	*	#	*	#	
AmelCav1a	VFQCVTLEGWTEVLY	348	VFQILTGEDWNAVMY	679	
HsapCav1.1	.Y..I.ME...D...	299	...V...E..TSM..	621	
HsapCav1.2I.ME...D...	341E...S...	684	
HsapCav1.3I.ME...D...	371E...A...	732	
HsapCav1.4V.ME...D...	337E...V...	707	
AmelCav2a	VFQCITMEGWTAILY	285	VFQILTGEDWNEVMY	632	
HsapCav2.1E...DL..	325E...E...	675	
HsapCav2.2E...D...	321E...A...	670	
HsapCav2.3E...TV..	316E...E...	664	
AmelCav3a	IFLVISLEGWTDIMY	412	VFQILTQEDWNVVLF	1063	
HsapCav3.1	..Q..T.E..VD...	361E...K..Y	930	
HsapCav3.2	..Q..T.E..VD...	361E...V..Y	930	
HSapCav3.3	..Q..T.E..VE...	364E...V..Y	793	
	Loop III		Loop IV		DCS locus
	*	#	*	#	
AmelCav1a	LFTVSTFEGWPSSLDD	1111	LFRSATGESWQEIMM	1399	EASE
HsapCav1.1E...Q..Y	1021	...C...EA..E.LL	1330	DSQE
HsapCav1.2E...E..Y	1093	...C...EA..D..L	1394	DSED
HsapCav1.3E...A..Y	1128	...C...EA..E..L	1433	DAAE
HsapCav1.4E...A..Y	1082	...C...EA..E..L	1379	DVAE
AmelCav2a	LFAVQTGEGWPQILQ	1008	LFRCATGEAWPNIML	1296	AEQN
HsapCav2.1	..T.S..E...QV.K	1467	...S...E..HN...	1763	DEQN
HsapCav2.2	..T.S..E...MV.K	1372	...S...E..HE...	1662	DAME
HsapCav2.3	..T.S..E...QV.Q	1378	...S...E..QE...	1669	TEQE
AmelCav3a	LFVLSSRDGWVNIMY	1740	LFRVATGDNWNGIMK	2037	DVNG
HsapCav3.1A.KD...D...	1470S..D...G...	1775	DKDG
HsapCav3.2A.KD...D...	1470S..D...G...	1786	DVDG
HSapCav3.3A.KD...N...	1352	..Q.S..D...G...	1650	EVNG

Fig. 6. Alignment of the P-loops of honeybee and human Cav α subunits. The human sequences used for this alignment are the same as in Fig. 4. The amino acids of the EEEE (*) and the DCS (#) loci are highlighted in bold. The sequence of the DCS locus of each Cav α subunit is shown at the bottom on the right. Dots represent identical amino acids.

Buraei and Yang, 2010). The GK-AID interaction is sufficient to allow Cav α membrane trafficking and, consequently, for increasing current amplitude. The GK-SH3 interaction is important for the specific gating regulations provided by the different Cav β subunits. In particular, the HOOK domain and the N-terminus play a predominant role in modulating channel inactivation (Cens et al., 1999; He et al., 2007; Miranda-Laferte et al., 2012; Olcese et al., 1994; Qin et al., 1996; Restituito et al., 2000; Richards et al., 2007). The sequences of these two regions are extensively spliced in the mammalian Cav β subunits (Buraei and Yang, 2010). Here, we clone AmelCav β c (Genbank: JX997993), a third variant of the honeybee Cav β subunit that shares exon 1a with AmelCav β a but lacks exon 9, like AmelCav β b (Supplementary Fig. 2). Compared to AmelCav β a and AmelCav β b, AmelCav β c has a longer exon4 that adds 12 amino acids to the HOOK domain through the use of an alternative donor site (Fig

7B). Mutually exclusive splicing of exon 7 of the mammalian Cav β 1 and Cav β 2 subunits spanning the 3' end of the HOOK domain leads to a V2 sequence of variable length because exon 7a adds 44 amino acids, including a polybasic domain also found in honeybee Cav β (Cens et al., 2013), while exon7b adds only 6 amino acids (Buraei and Yang, 2010). In *L. stagnalis*, exon 7, spanning the HOOK domain, can be skipped altogether or, via the use of an alternative acceptor site, can lead to a V2 sequence containing seven extra amino acids (Dawson et al., 2014). In *A. mellifera*, like in mammals (He et al., 2007; Richards et al., 2007) and snails (Dawson et al., 2014), the HOOK domain may work together with the N-terminus to regulate channel inactivation. The availability of the whole repertoire of Ca²⁺ channel subunits will undoubtedly be an asset to assess the functional consequences of Cav β alternative splicing on the various honeybee Ca²⁺ channels.

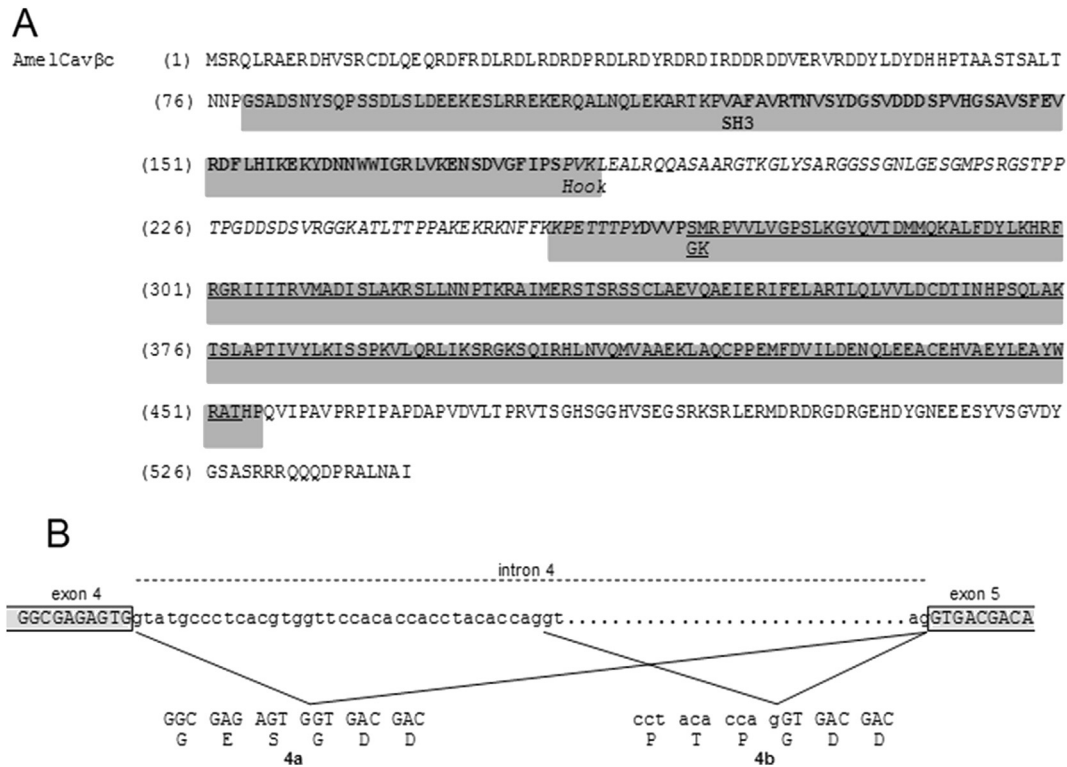


Fig. 7. Amino acid sequence of the AmelCav β c subunit. A. The two conserved C1 and C2 domains are highlighted in light gray, the SH3 in **bold**, the HOOK in *italic* and the GK sequence is underlined. B. Schematic representation of the alternative use of the donor site at the junction between exons 4 and 5 of AmelCav β c, based on the Genbank genomic sequence NC_007082.3. The AmelCav β c sequence was deposited in Genbank (accession number JX997993).

3.4. Molecular cloning of the three honey bee Cav α 2 δ subunits

The mammalian genome contains four genes encoding four different Cav α 2 δ subunits. Cav α 2 δ subunits are post-translationally cleaved to produce an extracellular, heavily glycosylated, α 2 peptide and a membrane-associated δ peptide. These two peptides are linked through a disulfide bond (see for review Dolphin, 2013). The site of the proteolytic cleavage has been identified only in the Cav α 2 δ 1 subunit, but the involved protease remains unknown (Andrade et al., 2007). Similarly, the cysteine residues that participate in the formation of the disulfide bond have been recently identified in the sequence of mammalian Cav α 2 δ 1 (Calderón-Rivera et al., 2012), but other cysteine residues could be involved in the other Cav α 2 δ subunits. Moreover, little is known on how Cav α 2 δ affects channel properties, because the interaction between Cav α 1 and Cav α 2 δ has only been partially unraveled (Dolphin, 2013). Cav α 2 δ C-terminus contains a hydrophobic domain that is predicted to be a helical trans-membrane segment (Calderón-Rivera et al., 2012). However, this would leave a very short intracellular sequence, particularly in the case of the Cav α 2 δ 4 subunit (only a single amino acid). Alternatively, Cav α 2 δ might be glycosyl-phosphatidylinositol (GPI)-anchored (Davies et al., 2010). The α 2 peptide contains protein domains including a von Willebrand factor A (VWA) domain generally involved in protein–protein interactions and a Ca²⁺ channels-chemotaxis receptor (cache) domain also found in bacterial chemosensing proteins. While the function of the VWA domain has been partially identified by mutating its metal ion-dependent adhesion motif (MIDAS), the functional role of the cache domain is unknown (Dolphin, 2012). On the basis of the predicted cDNA sequences (Genbank: XM_003251054, XM_003251053 and

XM_623846), we cloned and arbitrarily numbered three *A. mellifera* Cav α 2 δ subunits. None of these sequences showed high homology with a specific mammalian Cav α 2 δ subunit (Table 2). The genomic sequences of AmelCav α 2 δ 1, α 2 δ 2 and α 2 δ 3 contain 17, 12 and 22 exons, respectively, that are predicted to span 9–10 kb (Supplementary Fig. 3). AmelCav α 2 δ 1, AmelCav α 2 δ 2 and AmelCav α 2 δ 3 cDNAs (deposited in Genbank under the numbers KJ485707, KJ485708 and KJ485709) encode proteins of 1204, 1198 and 1193 amino acids, respectively (Fig. 8). We did not find any evidence for the existence of alternative exons for any of the three Cav α 2 δ subunits. All Cav α 2 δ subunits contain a signal peptide at the N-terminus, in agreement with their predicted exofacial N-terminus, and a hydrophobic domain at the C-terminus (Fig. 8). If this hydrophobic domain was a trans-membrane segment then the cytoplasmic C-terminus of AmelCav α 2 δ 1 would be 5-amino acid long, whereas that of AmelCav α 2 δ 2 and AmelCav α 2 δ 3 would be non-existent (Fig. 8). To assess whether the honeybee Cav α 2 δ subunits could be GPI-anchored proteins like some of the mammalian Cav α 2 δ subunits (Robinson et al., 2011), we analyzed their sequences using three independent algorithms: Big-PI (Eisenhaber et al., 1999), FragAnchor (Poisson

Table 2

Sequence homology between honeybee and human Cav α 2 δ subunits.

% Amino acid identity	HsapCav α 2 δ 1	HsapCav α 2 δ 2	HsapCav α 2 δ 3	HsapCav α 2 δ 4
AmelCav α 2 δ 1	23	22	31	29
AmelCav α 2 δ 2	23	24	28	29
AmelCav α 2 δ 3	22	22	29	30

Genbank Accession number of the human (Hsap) subunits: Cav α 2 δ 1 (NP_000713), Cav α 2 δ 2 (NP_006021), Cav α 2 δ 3 (NP_060868) and Cav α 2 δ 4 (NP_758952).

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amCavα.281 (1) -----MRPRFLPVIVILILIEALVRGDSI SYNTVKTWANKLGFELSQLGKVFVN-----ADKFND SYKQAVLKPRD
amCavα.282 (1) ----MERIFDNRLIILAICLLCTDCLAKDSYIVTRWAEILGAELWELAEKVARPEELLISKYKAMNTRVENKSGE
amCavα.283 (1) MFLSVKKFVHYGVLLLLWIKSSYQOEE DIPHNVEKNWALKFGVDLWE FGRQVTKMSE IQRKYHDMEEAEVVKKDG-

(67) GNALVHEIAKDIKAMMESKISAIKRIMDVAETSALSAPDVPPESEFNYTNAKNNTIDLK-----
(72) --KLVNII SENVGRMLRRKMDAVTCIRMAAEEYAEENWENDEEGNFTYVSGKYSQVMNTRTRPRI PKNMKKNID A
(75) -VLLVREMAAEVKNMLDFKMNAMVRLVE SAEQAASAPRDNVSPKYYASQRF DSSSGE-----GKASIT

(126) -----HSAHFGGQVNLDRSAVHVP INYVDRASNV IRAIKWSEELDKTFINNYEQDPSLSWQYFGSATGFMRO
(145) YRKMELTSDSHFYNI PVNTSFS SVHIPTINVYDMSPPVNDIKKTEILDNI FRQNYES DPALSWQYFGSVTGMLRQ
(139) VQETFLS SNRHF DHLAVNVTLS TVLLP DGVKQ IDREVAAGIQWSEYLDLLFVNYES DSSLSWQYFGATSGFLRR

(193) YPAMNWMYEP-----VDLFDCRTRSWYIEAATSPKDIILIDITSGSM
(220) YPAMEWKTNPTEIISADKAEDDEKSEDKDKNKDEEEDEKEEADIYDCRVRSWFIEAATCSKDMVILMDITSGSM
(214) FPAISWPVNDRAY-----GADKNRAIRDVYEFRIISDNFVGAANSFKDLALIDIECYA
VWA * * *
(235) TGI RREI ARHVNNILDTLGNNDFVNI I TFSNVTKEVVPFCFNDTLVQANLANVRELKRAILNLDTEKIAN-FSLA
(295) TGMGKTIARTIVSVILD TLSNND FVTLSYANETYDVPVPCFKDMLIQATPENVDTFKKALIDVKTGEGLAN-LTEA
(268) SERNKRLAVITVKTILDTLGPNDYVNIYRYGDTAEIIVQCFKDSLVOA SPENVHDLKI AMSSMKHEEIP TNISAA
*

(309) LTAFELLETYRTEREG---ARCNOAIMLITDGVFPYNYKEIFETYNWRDNPDEPFKADMPVRFMFTYLIGREVADV
(369) FTKAFSLNNTYRETRGC GADTPCNQLIMLVTDGVPGNLTFVFKTWNWRENDTH-----IPVRVFTYLLGKEVTKV
(343) LATAFEILHRYNRIGQGSQ---CNOAIMLITADNAGLPTIEVIKRYNWP-----MPVRIFTYLLIGGDKS--
*

(381) KEVQWMA CANRGYFVHLCTLAEVREEVLKYVPVMARPLVLGR TDHPTIWTVPYADVTDPKMTDWLWEQRESEEQK
(439) REIQWMA CLNRGYTHVHTLEE VREQVLKYI PVVARPMVLQEVVHPVWTHAYADITLDKDE DVRQDA-----
(404) PELRNTACANKGFYARITLEDIRS KVFYVVKVLARPMVL YQHEHPIHWS FVYVGGKSSRYGKE-----
#
(456) ERFLNLHKRRKLLNSEERDRRFVKKQKSHDQSGDLQEYRLMITSVSI PVFDRREN---ATRIADLLGVAGTDVPI
(507) -----SLNNTAWQEYRLLTSVGT PVFDRKGNRNRTRMANLLGVAGTDVPI
(468) -----NIGQIMTSVIAFILDRRNYT---VKIANLLGIVGTDVFP
Cache
(528) EEIQKLMMPHLLGVNGYAFIVTNGGFI LHPDLRPFVFG-----ILKPAYNSVDMAEVELMDQDKEPRE-FDEGI
(554) DDIRKTLIPYKLGVNGYAFIVSNGGIVLHPDLRPFVFG-----KLKLNYSVDLTFEVELDGRGPRNPGPE-V
(504) EEIQKLVFPYKLGVNGYFIVDNNGRVLYHPDLRPLPGNI DYEETLKPTYISVDLSEVELAEYDGLHPLNNSLL

(597) IMLRNDVNVQONG-SVTLHTKYHYDDMKRVGR IKRKYDFTIGI PKTPTVIVSLPEHDHTGNRVHAT EEIHRSHV
(623) LELRGALVDHKS GSKSVKLVHYDNNRRVILEKRDY YAPLPGT PFG LAVAMSSS-NYKGTWIKVGD EIRRNQN
(579) LDLRRDMIDQKEG-ETNFAIKIHYDNMQRVTIRRHNYFYKSI EGT PFGSLGLALPEG-YGMFELLAEQEIKAHAIIN

(671) SGINVSDYFAGTNRVH PHLWYCKYHYEDERS FNSSE AQLLH FLERTRQPRWKNWDMKQPSQPPEYSATNSGNET
(697) MNVNISE FVGNRVRVH PSWYCRYHYLEGHE FDNPEEELRH FLNLLNKPGWKWSEQYEAQIDVNE TNYVPCG
(652) V----TEYFKGNWVKVH PDWVYCEYSSASEKWFPSPFERVLH FLTRTRSPGWKWSLRRPSPSSHKQASKPDK-

(746) HRKSKPTPYKIDKDSYYCDRDL LSLVFDKAVTQWFANLNI TREE-KAKTFQQRFGVTLAFMATHSGLTRWQDFL
(772) RQT-----LSHDDYYCNKELMQLLV FDKA TNASFNDVLDLDDAR TRNLT HVYGVFLRFVATQSGLTRWHYLD
(722) -----DAYYCDKKLVSQSLVDLALVTDGFNKRGMHKEENQNGTSTFGVTRSFITRSGLFRWHEHQ

(820) LDEEGVIP---DDHFSKMPRA IDEVWYKRAVEQYVQPESEFVFSVP IDEG-ADNTTLVTASRAIFI DTERAKAP
(840) TNKLPEDNDG--IVFGDLHRKAVNEPWYKAAI FQNTLDPNSI SLSVPEAG--PDAIVTVS IGLFPKDGGRKRAA
(784) QNTEDNTDESP--FAEKYARAMDSSWYKRAVDQHSI EPDSFVFSVPFN--AADSPNPLVTATHAVFIGTGHKAP

(891) VAVVGFQFQHTALQGLFQNI TSCGSGKCHT HCGADNWACYLIDNNGYVIAAKDKS--DAGKFFGLRGPIMSS
(910) AAVIGFQMPMTNLHDKFIELTSKSNST--LMCAHVWIDCYLLDQNGFVVI SEAHN--NTGQFMGTQEGAVMSS
(854) AAVVGLQFQHSSLASRFVNITSTCSGTN-CKKNCASDALDCYILDNNGFIIISER--HEHTGKFFGEIDGTIMDS

(964) LVKEGVFERIRI FDYQAVCFKST-QTSNDGSI LLAPWKHAQKMI SWLIGQAVWAWAKAGIWESEYAYAYPNEDED
(981) MVGQGLYNPIEIYDYQAWCEEVR--IEAAANTLTDPLVYIWKLLLWI LLRFTWFTTQFVNLPISYAKVHFDE DAP
(926) LVQDRIYRKVTVTDYQGICSPQESHQS SASRTFSESVAKTIA ILGNFLWSMAFGFNFQNLWQVAFAFAGESVRPL

(1038) IHEEYQDNDQE-----K-----PPITEKLFQKVLINRTRPEACDQEVYLYLRNAS
(1054) DPPPPPRP-----YLYHYPCDQKRILYMMNTT
(1001) DDSIGQVHEFE SLAIDGGGEP TDEPI SDGNF PRLPT ITAAT PASPGTTRATSTHHLRTRLR SCKKTDLYILQPD

(1084) FDMFDIDS-----DVKK-DCMRPYIVQPVNYSNMLLVVNTACTETTMP----PLSVI PQEIIYENNSLVCQ
(1081) IASQGITN-----HSDY--CSRPFYARRVPHNLLLWVDSMYPICYKRLEVT PVNISPLEYNTGESAPCH
(1076) RLNTSGQSNPLKGLTNCHDTGCERPFSVQKIPHTNLILLVVDLTLPCGSK-----QLSIEPIEALTEPG--ACI

(1146) KALISLKRKR-PQSCIRSHSRESEIKDLCGLASNVTPNIYLFLLSITCSLIQRI DLGQH
(1146) KIPLNDLKRRLLEGCFTEHPLEY-EIEDCGGASGLTVSLLLFSTAIARI LYTFV-----
(1144) ARRELRYRRR-PPKCI NYHPEEM-EIKFCGSANRPFPLFIVAIVSSTLA-----
# ω ω

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Fig. 8. Alignment of the amino acid sequences of the three AmelCav α 2 δ subunits. Dashes represent gaps introduced to maximize sequence alignment. The signal peptide at the N-terminus (*italic*) and the trans-membrane segment at the C-terminus (**bold**) were predicted with SignalP4.0 and TMbase on the ExPASy server (www.expasy.org), respectively. The VWA domain, which includes the MIDAS site (*), is underlined. The two cysteine residues involved in the formation of the disulfide bond in mammalian Cav α 2 δ 1 are marked (#). The potential GPI-anchor sites C1170 and K1168 (ω) were identified with bigPI and PredGPI, respectively. Sequences were deposited in Genbank (accession numbers KJ485707–KJ485709).

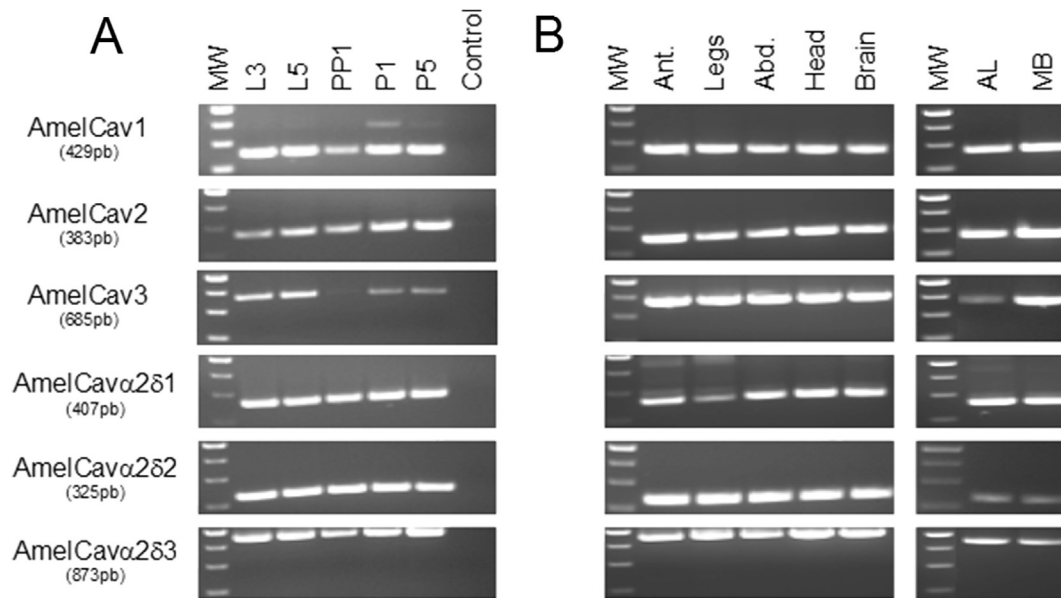


Fig. 9. Developmental and tissue specific expression of the AmelCav α and AmelCav α 2 δ subunits. Total RNA was extracted from whole honeybees at different developmental stages: larva (L3 and L5), pre-pupa (PP1) and pupa (P1 and P5) (A), or from adult tissues: antenna (Ant.), legs, abdomen (Abd.), head, brain, antennal lobes (AL) and mushroom bodies (MB) (B). Note that there is a faint band of the expected size for AmelCav3 at the PP1 stage.

et al., 2007) and PredGPI (Pierleoni et al., 2008). The three programs (Supplementary Table 2) predicted that AmelCav α 2 δ 1 was not GPI-anchored and that AmelCav α 2 δ 3 is GPI-anchored, but the site of post-translational modification predicted by Big-PI is different from the one predicted by PredGPI (both indicated by ω in Fig. 8). Conversely, only two programs predicted that AmelCav α 2 δ 2 was GPI-anchored. Similar discrepancies were also reported for the mammalian Cav α 2 δ subunits (Robinson et al., 2011).

3.5. Expression of Ca²⁺ channel subunits during development and in adult honeybees

To evaluate the physiological functions of Ca²⁺ channels in honeybees, we first checked the expression of the different subunits during development and in different tissues from adult bees by RT-PCR (Fig. 9). The three AmelCav α and the three AmelCav α 2 δ subunits were expressed from the larval stage and throughout development, suggesting that Ca²⁺ channels may be involved in bee pre-imaginal development. Compared to the different subunits, AmelCav3 varied most during development, but this needs to be more precisely assessed. All subunits were also expressed in all adult tissues assessed, including legs and brain, and more precisely, they were all expressed in sub-regions of the brain namely the antennal lobes and the mushroom bodies. Knowing the central role of Ca²⁺ signaling in multiple cellular functions, the ubiquitous expression of all honeybee Ca²⁺ channel subunits is not surprising.

3.6. Functional expression of the honeybee Ca²⁺ channels

To our knowledge, Ca²⁺ currents resulting from the expression of insect Ca²⁺ channel subunits in a heterologous expression

system have never been recorded. This could be due to the lack of one or more auxiliary subunits in previous experiments, as reported for squid Ca²⁺ channels (Kimura and Kubo, 2002). We therefore injected in *Xenopus* oocytes a mixture of *in vitro* transcribed RNAs encoding AmelCav1a or AmelCav2a together with those encoding AmelCav β c and AmelCav α 2 δ 1 (Fig. 10). Even in these conditions, however, the expression of Ca²⁺ currents was rarely observed and frequently we did not record any current at all in a whole batch of injected oocytes. This may suggest the existence of RNA secondary structure, post-translational modifications or unknown regulatory subunits that could chaperone Ca²⁺ channels to the plasma membrane. We obtained similar results by combining other honeybee Cav β and Cav α 2 δ subunits (not shown). Nevertheless, we could draw current–voltage curves for both AmelCav1a and AmelCav2a channels that clearly displayed distinct properties (Table 3). On the other hand, expression of AmelCav3a alone gave more reliable results. Compared to AmelCav1a and AmelCav2a, this channel was clearly transient with the fast inactivation kinetics described for mammalian Cav3 channels (Fig. 10A). This channel activated at around –50 mV and showed a more negative reversal potential than AmelCav1a and AmelCav2a channels (Fig. 10B and Table 3). We unambiguously conclude that AmelCav3 encodes a LVA Ca²⁺ channel. Nevertheless, improving the expression of these channels would be advantageous for a more complete characterization of their electrophysiological and pharmacological properties.

In conclusion, we have characterized all the voltage-gated Ca²⁺ channel subunits of the honeybee *A. mellifera*. They include functionally important protein domains identified in mammalian subunits. When expressed in *Xenopus* oocytes, they produce Ca²⁺ channels with distinctive properties. The availability of these subunits opens new opportunities for elucidating the role of Ca²⁺ channels in honeybee physiology.

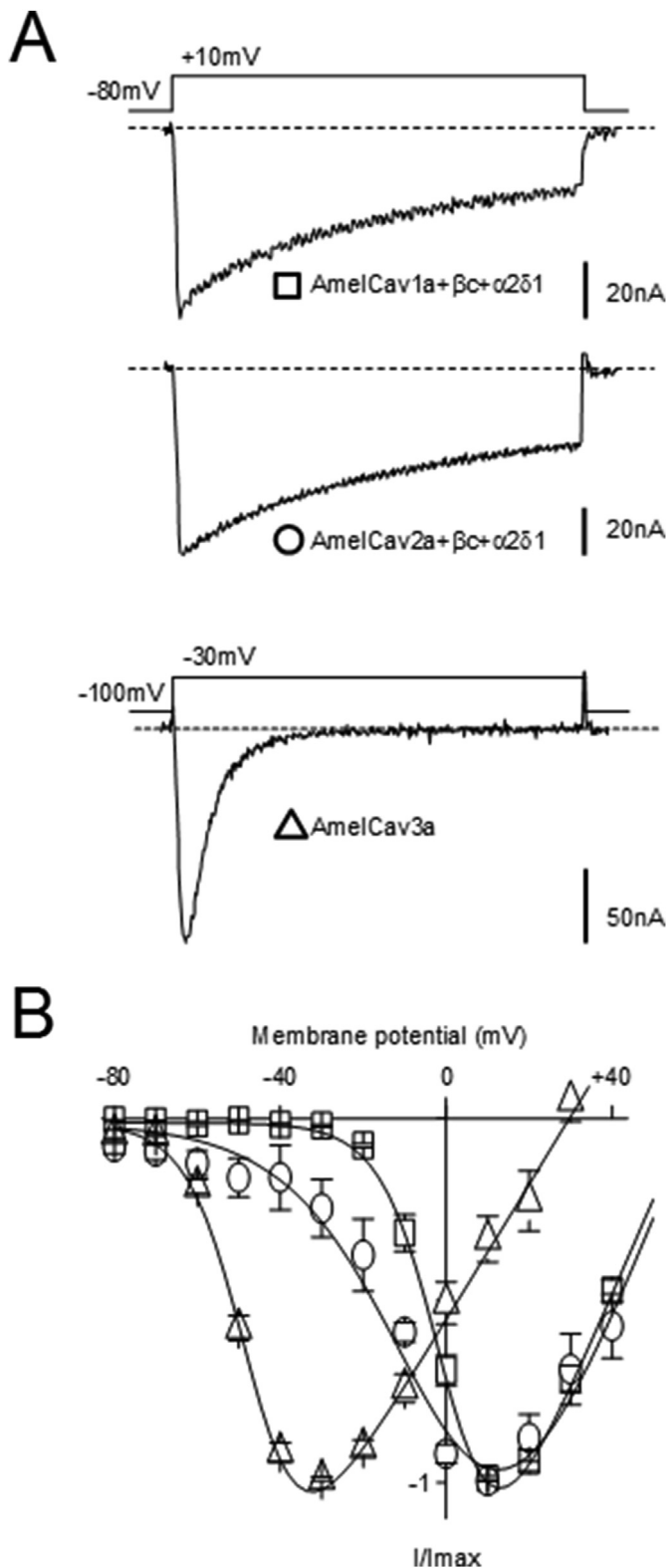


Fig. 10. Recordings of honeybee Ca^{2+} channel currents. **A.** Traces of currents obtained in *Xenopus* oocytes injected with a mixture of *in vitro* transcribed AmelCav1a (square) or AmelCav2a (circle) together with AmelCav β c and AmelCav α 2 δ 1, or AmelCav3a (triangle) RNAs. **B.** Current–voltage relationships obtained with the three subunit combinations. Note the fast inactivation and the hyperpolarizing shift of the current–voltage curve obtained with AmelCav3a.

Table 3

Parameters of the current–voltage curves obtained for honeybee Ca^{2+} channels.

	Va (mV)	k (mV)	Erev (mV)	n
AmelCav1a	5 ± 2	8.8 ± 0.9	56 ± 2	4
AmelCav2a	4 ± 6	14.2 ± 1.7	54 ± 5	3
AmelCav3a	46 ± 1	7.0 ± 0.4	26 ± 1	8

Va: potential for half activation, k: slope factor, Erev: reversal potential.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ibmb.2015.01.005>.

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