Divergent Ca^{2+}/calmodulin feedback regulation of Ca_V1 and Ca_V2 voltage-gated calcium channels evolved in the common ancestor of Placozoa and Bilateria

Julia Gauberg[1], Wassim Elkhatib[1], Carolyn L. Smith[2], Anhadvir Singh[1], and Adriano Senatore[1,*]

[1]Department of Biology, University of Toronto Mississauga, 3359 Mississauga Road, Mississauga, ON L5L 1C6, Canada
[2]NINDS, National Institutes of Health, Bethesda Maryland, 20892 USA.
*Corresponding author: Adriano Senatore
E-mail: adriano.senatore@utoronto.ca
Tel. (905) 569-4322

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Table S1. Reports of significant one-way and two-way ANOVA tests conducted throughout the study. The p-values of post-hoc tests associated with each ANOVA are indicated throughout the results.

Fig 2D: EGFP fluorescence

One-way ANOVA

Source of Variation	DF	SS	MS	F	P
Between Groups	4	1.402	0.351	45.965	<0.001
Residual	10	0.0763	0.00763		
Total	14	1.479			

Fig 4F: $V_{1/2}$ and Slope factor (k)

$V_{1/2}$ conductance – one way ANOVA

Source of Variation	DF	SS	MS	F	P
Between Groups	2	2376.352	1188.176	36.569	<0.001
Residual	22	714.816	32.492		
Total	24	3091.168			

$V_{1/2}$ steady state inactivation – one way ANOVA

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1054.616	527.308	59.127	<0.001
Residual	17	151.610	8.918		
Total	19	1206.225			

k conductance – one way ANOVA

Source of Variation	DF	SS	MS	F	P
Between Groups	2	13.789	6.894	6.883	0.005
Residual	23	23.038	1.002		
Total	25	36.827			

k steady state inactivation – one way ANOVA

Source of Variation	DF	SS	MS	F	P
Between Groups	2	218.406	109.203	44.847	<0.001
Residual	17	41.395	2.435		
Total	19	259.801			

Fig 6: τ activation

rCa_V1.2 – one-way repeated measures ANOVA

Source of Variation	DF	SS	MS	F	P
Between Subjects	10	2.547	0.255		
Between Treatments	12	50.486	4.207	90.368	<0.001
Residual	102	4.749	0.0466		
Total	124	57.607	0.465		

TCa_V1 – one-way repeated measures ANOVA

Source of Variation	DF	SS	MS	F	P
Between Subjects	11	20.311	1.846		
Between Treatments	15	523.176	34.878	68.928	<0.001
Residual	150	75.902	0.506		
Total	176	632.227	3.592		

TCa$_V$1 vs rCaV1.2 – two-way ANOVA

Source of Variation	DF	SS	MS	F	P
channel	1	52.252	52.252	205.015	<0.001
voltage	12	181.353	15.113	59.296	<0.001
channel x voltage	12	27.810	2.317	9.093	<0.001
Residual	248	63.208	0.255		
Total	273	343.786	1.259		

Fig 6: τ inactivation

rCa$_V$1.2 – fast τ – one-way repeated measures ANOVA

Source of Variation	DF	SS	MS	F	P
Between Subjects	11	2954.280	268.571		
Between Treatments	9	44357.454	4928.606	40.720	<0.001
Residual	83	10046.015	121.036		
Total	103	58816.921	571.038		

rCa$_V$1.2 – slow τ – one-way repeated measures ANOVA

Source of Variation	DF	SS	MS	F	P
Between Subjects	11	23246.960	2113.360		
Between Treatments	9	194487.117	21609.680	18.835	<0.001
Residual	75	86050.459	1147.339		
Total	95	311492.844	3278.872		

TCa$_V$1 – fast τ – one-way repeated measures ANOVA

Source of Variation	DF	SS	MS	F	P
Between Subjects	11	3340.943	303.722		
Between Treatments	12	32626.001	2718.833	33.372	<0.001
Residual	125	10183.700	81.470		
Total	148	46627.207	315.049		

TCa$_V$1 – slow τ – one-way repeated measures ANOVA

Source of Variation	DF	SS	MS	F	P
Between Subjects	11	30307.805	2755.255		
Between Treatments	12	238042.646	19836.887	10.991	<0.001
Residual	98	176875.725	1804.854		
Total	121	455990.051	3768.513		

TCa$_V$1 vs rCa$_V$1.2 – fast τ – two-way ANOVA

Source of Variation	DF	SS	MS	F	P
channel	1	15353.326	15353.326	159.662	<0.001
voltage	9	20541.683	2282.409	23.735	<0.001
channel x voltage	9	31366.891	3485.210	36.243	<0.001
Residual	201	19328.410	96.161		
Total	220	81762.689	371.649		

TCa$_V$1 vs rCa$_V$1.2 – slow τ – two-way ANOVA

Source of Variation	DF	SS	MS	F	P
channel	1	18038.487	18038.487	13.347	<0.001
voltage	9	110628.242	12292.027	9.095	<0.001
channel x voltage	9	108441.097	12049.011	8.915	<0.001
Residual	171	231114.230	1351.545		
Total	190	453886.248	2388.875		

Fig 6: τ deactivation

$rCa_V1.2$ – one-way repeated measures ANOVA

Source of Variation	DF	SS	MS	F	P
Between Subjects	5	0.0369	0.00739		
Between Treatments	9	1.115	0.124	32.466	<0.001
Residual	43	0.164	0.00381		
Total	57	1.319	0.0231		

TCa_V1 – fast τ – one-way repeated measures ANOVA

Source of Variation	DF	SS	MS	F	P
Between Subjects	15	5.581	0.372		
Between Treatments	9	21.243	2.360	8.727	<0.001
Residual	104	28.128	0.270		
Total	128	55.279	0.432		

TCa_V1 – slow τ – one-way repeated measures ANOVA

Source of Variation	DF	SS	MS	F	P
Between Subjects	11	5172.971	470.270		
Between Treatments	9	31281.409	3475.712	9.985	<0.001
Residual	70	24367.121	348.102		
Total	90	61080.477	678.672		

TCa_V1 (fast τ) vs $rCa_V1.2$ – two-way ANOVA

Source of Variation	DF	SS	MS	F	P
Channel	1	31.860	31.860	156.901	<0.001
Voltage	9	11.494	1.277	6.289	<0.001
Channel x Voltage	9	3.660	0.407	2.003	0.042
Residual	167	33.911	0.203		
Total	186	86.996	0.468		

Fig 7: TCa_V1 vs TCa_V2 pC/pA

Source of Variation	DF	SS	MS	F	P
channel	1	176.247	176.247	0.493	0.490
time	2	21446.271	10723.136	29.996	<0.001
channel x time	2	686.812	343.406	0.961	0.397
Residual	23	8222.086	357.482		
Total	28	31289.969	1117.499		

Fig 8G: R(+) BayK

R(+)-BayK 8644 – two-way ANOVA

Source of Variation	DF	SS	MS	F	P
channel	1	3.599	3.599	557.338	<0.001
concentration	4	4.132	1.033	159.970	<0.001
channel x concentration	4	0.758	0.189	29.331	<0.001
Residual	51	0.329	0.00646		
Total	60	8.759	0.146		

Fig 9D: r250- EGTA vs BAPTA

TCa_V1 – one-way ANOVA

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0.817	0.409	101.449	<0.001
Residual	19	0.0765	0.00403		
Total	21	0.894			

TCa_V2 – one-way ANOVA

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0.214	0.107	35.932	<0.001
Residual	20	0.0595	0.00298		
Total	22	0.273			

Fig 10: CaM WT vs CaM_{1234}

$rCa_V1.2$ – two-way ANOVA

Source of Variation	DF	SS	MS	F	P
CaM	1	2.501	2.501	360.440	<0.001
voltage	17	6.765	0.398	57.356	<0.001
CaM x voltage	17	3.148	0.185	26.684	<0.001
Residual	235	1.631	0.00694		
Total	270	15.393	0.0570		

TCa_V1 – two-way ANOVA

Source of Variation	DF	SS	MS	F	P
CaM	1	5.264	5.264	390.647	<0.001
voltage	17	12.335	0.726	53.845	<0.001
CaM x voltage	17	1.594	0.0938	6.960	<0.001
Residual	355	4.784	0.0135		
Total	390	27.965	0.0717		

TCa_V2 – two-way ANOVA

Source of Variation	DF	SS	MS	F	P
CaM	1	2.034	2.034	454.139	<0.001
voltage	17	10.064	0.592	132.167	<0.001
CaM x voltage	17	1.451	0.0854	19.059	<0.001
Residual	222	0.994	0.00448		
Total	257	16.126	0.0627		


*NSCaTE
*NATE
*Key cysteine
*DI S4
*Point mutations in CaV1 channels that hyperpolarize voltage-dependency
*DI P-loop
*AAs for Ca2+ selectivity
*AID
*Mutated CaV1.2 residues that hyperpolarize voltage of inactivation
*DII S4
*DII P-loop
*CaVI II-III linker atigen
*DIII S4
*DIII P-loop
*CaV1.2 exon 33
*DIV S4
*DIV P-loop
*EF-hand
*pre-IQ
*IQ
TCa_1
rCa_1.2
rCa_1.2
SrCa_1/2
TCa_3

Figure S1. Protein alignment of full-length Ca_V channel protein sequences from *Trichoplax adhaerens* (TCa_V1 to TCa_V3), rat ($rCa_V1.2$) and the choanoflagellate *Salpingoeca rosetta* ($SrCa_V1/2$). The alpha interaction domain (AID) is highlighted in *green* (with *black* text), positively charged arginine/lysine residues in segment 4 (S4) voltage-sensing regions of each domain are highlighted in *red*, the negatively charged glutamate/aspartate P-loop selectivity filter residues are highlighted in *purple*, and the calmodulin binding sites (EF-hand, pre-IQ, and IQ domains) are highlighted in *blue*. Amino acids that form the N-terminal spatial Ca^{2+} transforming element (NSCaTE) motif, the NSCaTE associated transduction element (NATE) motif, and the TCa_V1 II-III linker region used as an antigen for antibody synthesis are highlighted in *yellow*. Residues associated with point mutations that alter voltage-sensitivity of mammalian Ca_V1 channels are highlighted *orange* (106), and negatively charged residues suggested to contribute to Ca^{2+} selectivity are highlighted in *pink* (32). The optional exon 33 encoding a portion of the DIV S3-S4 loop in $Ca_V1.2$ channels is in *green* (with *white* text) (108).

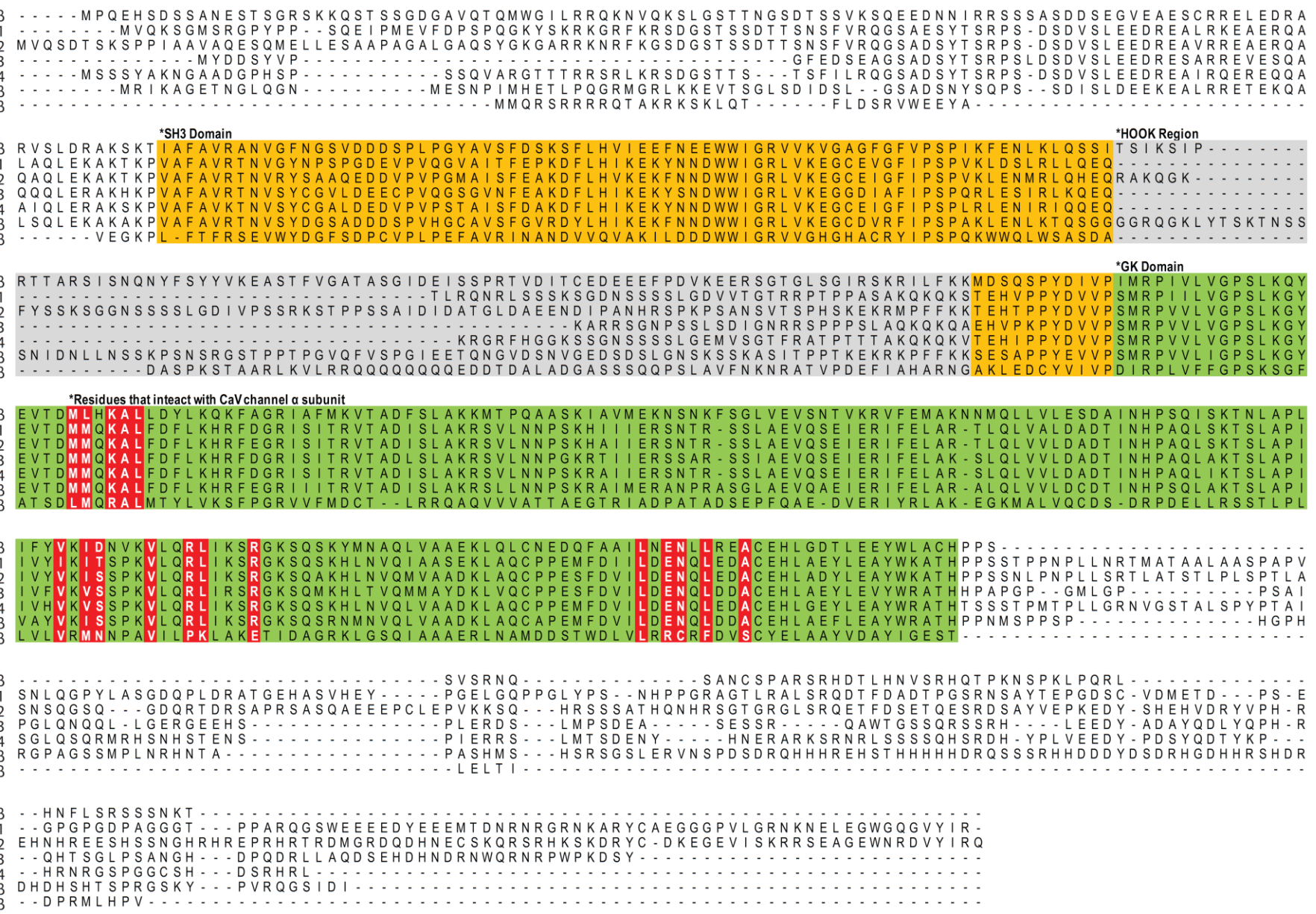
*SH3 Domain
*HOOK Region
*GK Domain
*Residues that inteact with CaV channel α subunit

Figure S2. Protein alignment of $Ca_V\beta$ protein sequences from *Trichoplax adhaerens* (*Ta*β), the rat *Rattus norvegicus* (*Rn*β1-4), the snail *Lymnaea stagnalis* (*Ls*β), and the choanoflagellate *Salpingoeca rosetta* (*Sr*β). The SH3 domain is highlighted in *orange*, the HOOK region in *grey*, and the Gunalyate Kinase (GK) domain in *green*. Amino acids identified in the rat β3 protein sequence that interact with the $Ca_V1.2$ channel AID region through x-ray crystallography are shown in red (38). Accession numbers for the included sequences are provided in File S1.

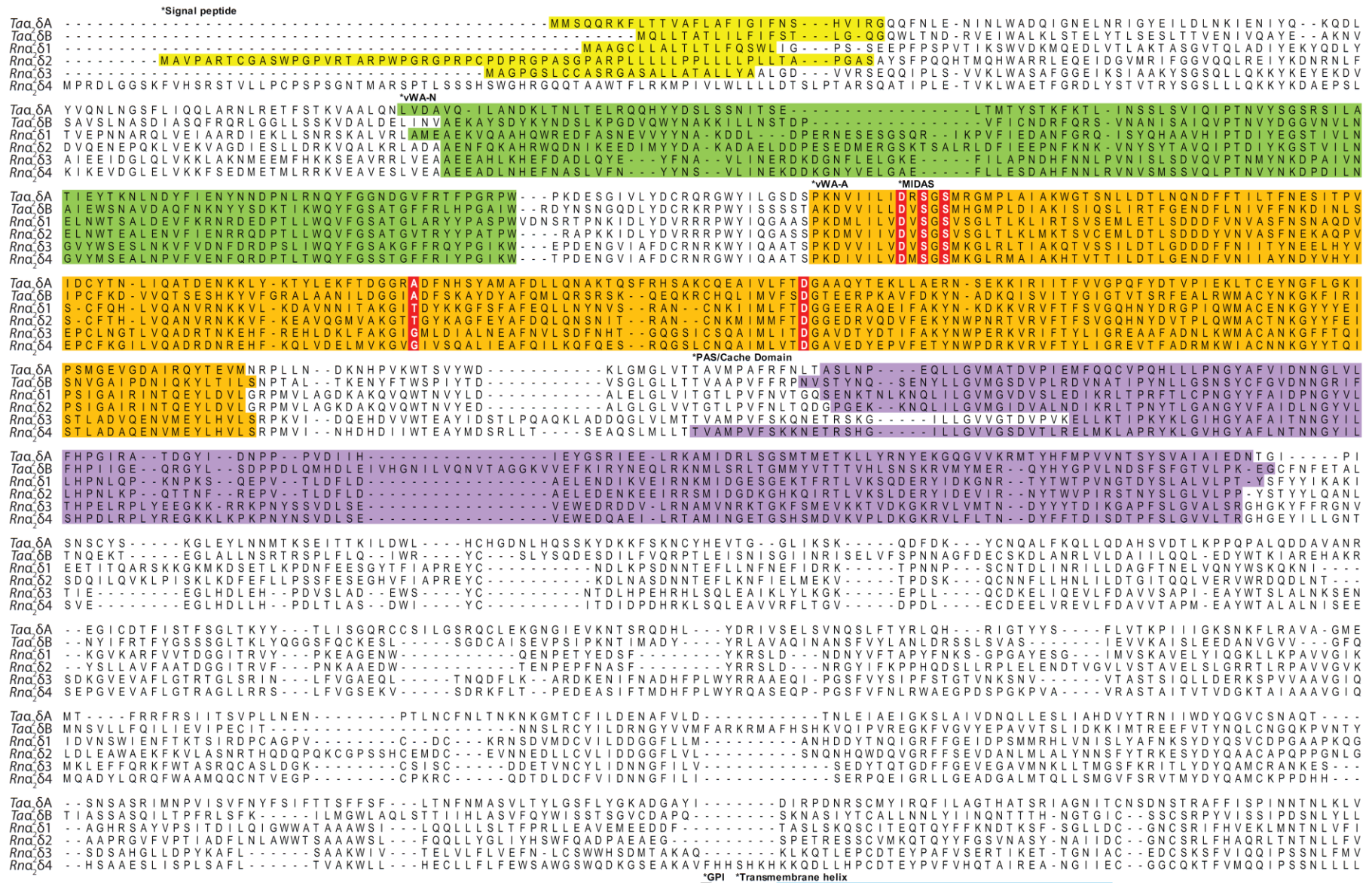
*Signal peptide
*vWA-N
*vWA-A
*MIDAS
*PAS/Cache Domain
*GPI
*Transmembrane helix
Taa δA
Taa δB
Rna δ1
Rna δ2
Rna δ3
Rna δ4

Figure S3. Protein alignment of $Ca_V\alpha_2\delta$ protein sequences from *Trichoplax adhaerens* (*Ta*$\alpha_2\delta$A and *Ta*$\alpha_2\delta$B), and *Rattus norvegicus* (*Rn*$\alpha_2\delta$1-4). Protein sequences bearing domains/sites predicted with InterProScan are highlighted as follows: signal peptides in *yellow*, von Willebrand factor type A-N (vWA-N) domains are in *green*, vWA-A domains are in *orange*, PAS/Cache domains are in *purple*, and C-terminal transmembrane helices are in *cyan*. Metal Ion-Dependent Adhesion Site (MIDAS) residues are highlighted in *red*, and C-terminal cysteine residues for extracellular glycophosphatidylinositol (GPI) anchoring are highlighted in *grey*. Accession numbers for the included sequences are provided in File S1.

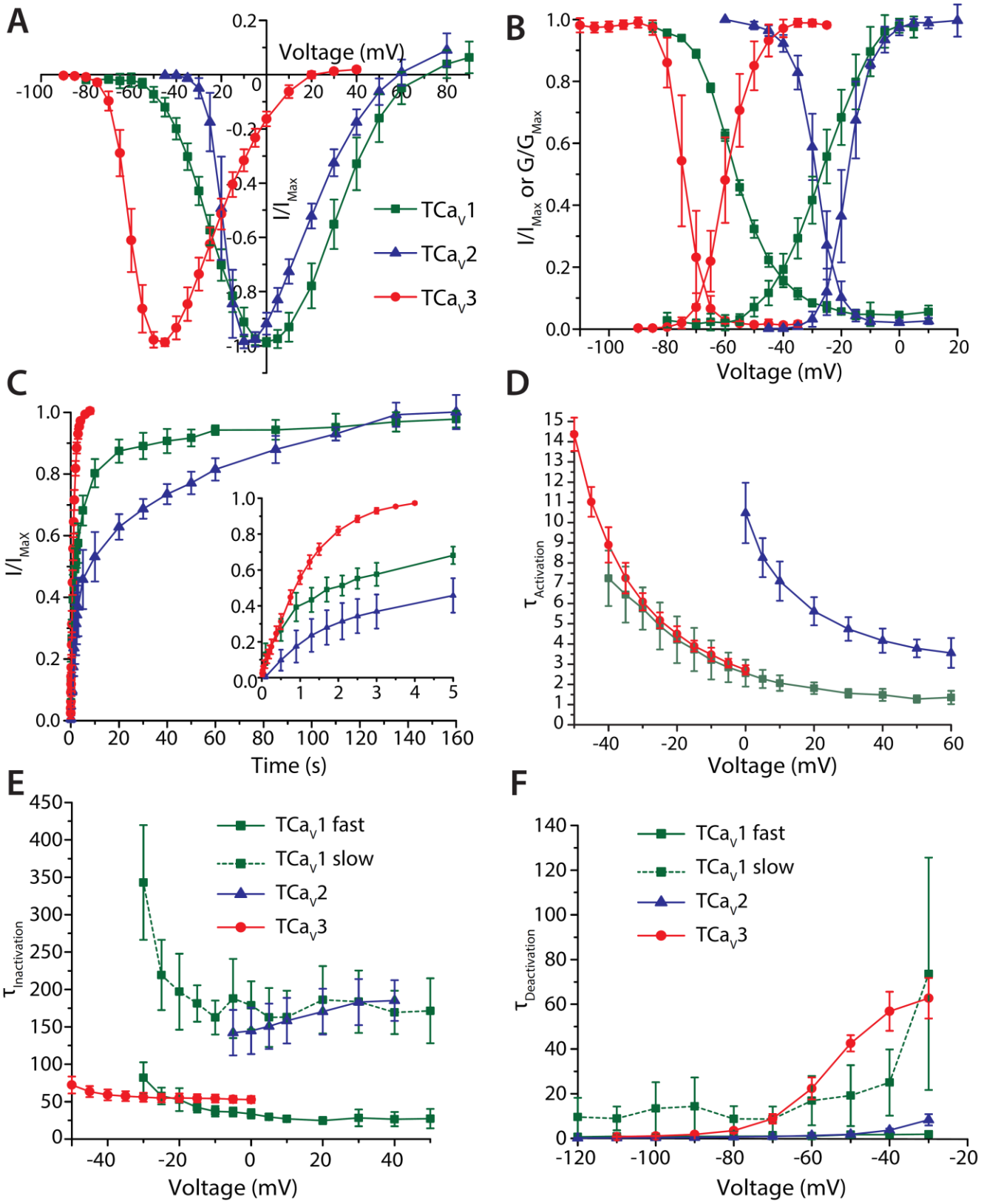

Figure S4. Comparison of voltage and kinetic properties of the *Trichoplax* Ca_V channel set. ***A***, Current-voltage relationships ± S.D (*error bars*) for TCa_V3 (*red*), TCa_V1 (*green*), and TCa_V2 (*blue*) reveal conserved bimodal classification as low voltage activated (TCa_V3) and high voltage activated (TCa_V1 and TCa_V2), with

respective maximal inward currents occurring at -45, -10, and 0 mV. ***B***, Plots of average inactivation and conductance ± S.D. reveal that TCa_V1 voltage properties are intermediate relative to TCa_V2 and TCa_V3. ***C***, Plots of average recovery from inactivation ± S.D. *Inset*: current amplitude recovery data for the first 5 s, with inflections in the curves for TCa_V1 and TCa_V2 indicative of two-component recovery from inactivation for both channels, compared to a single component for TCa_V3. ***D***, Plot of average $\tau_{Activation}$ values ± S.D. over depolarizing voltages. $\tau_{Activation}$ values were obtained by monoexponential curve fitting over the activation phase of macroscopic Ca^{2+} currents. Notable is that TCa_V1 and TCa_V3 activation kinetics are overlapping at common voltages, and both are faster than those of TCa_V2. ***E***, Plot of $\tau_{Inactivation}$ values ± S.D. obtained by curve fitting over the inactivation phase of macroscopic Ca^{2+} currents elicited by different depolarizing voltages. TCa_V1 fitting produced two components for inactivation, with a fast component comparable to that of TCa_V3, and a slow component comparable to TCa_V2. ***F***, Plot of average $\tau_{Deactivation}$ ± S.D. obtained by curve fitting over tail currents elicited by hyperpolarizing voltage steps. TCa_V1 fitting produced two components for deactivation, with a fast component similar to the single component of TCa_V2 deactivation, and a slow component that resembled TCa_V3 deactivation in its deceleration at more depolarized voltages.

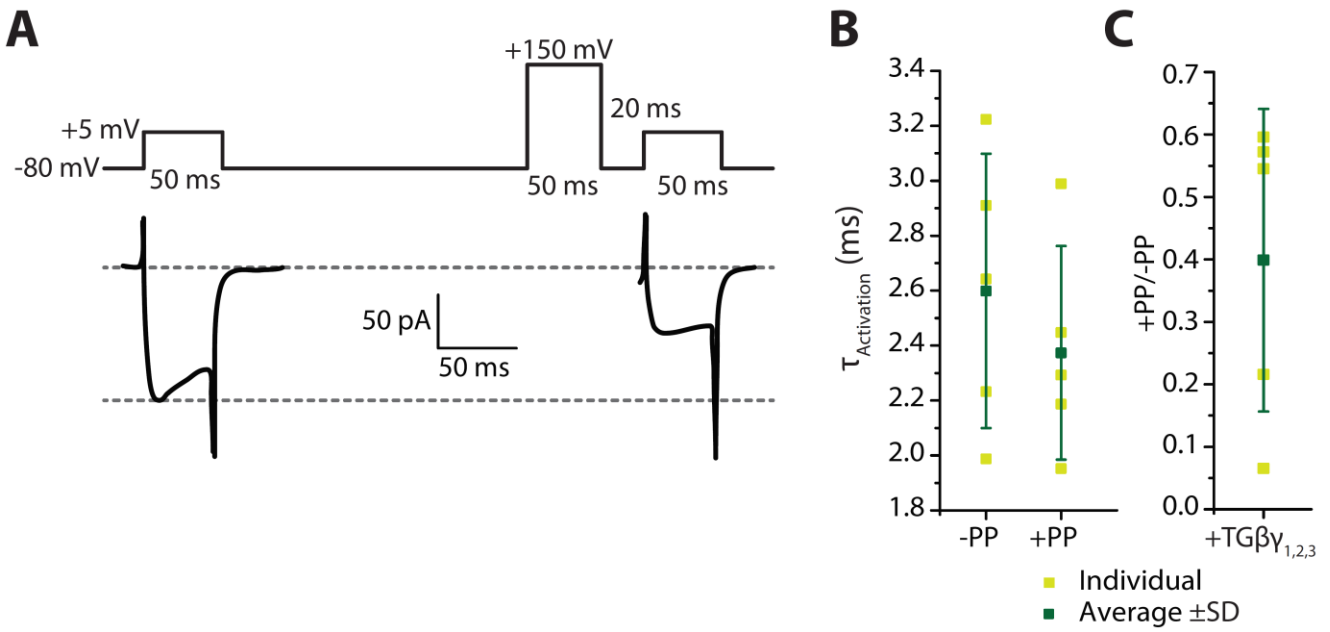

Figure S5. Absence of voltage-dependent Gβγ inhibition for the TCa$_V$1 channel *in vitro*. ***A***, illustration of the voltage-clamp protocol used to assess G-protein inhibition of TCa$_V$1 channels *in vitro* (top), with a sample current trace elicited by voltage steps to +5 mV before and after a strong depolarizing pre-pulse to +150 mV shown below. ***B***, Average $\tau_{Activation}$ values ± S.D. (*error bars*) before and after a +150 mV pre-pulse (PP) reveal no difference in activation kinetics. $\tau_{Activation}$ was calculated by monoexponential curve fitting over the activation phase of TCa$_V$1 macroscopic currents. ***C***, Average facilitation of peak macroscopic current amplitude after the pre-pulse ± S.D in cells co-transfected with *Trichoplax* G$\beta_1\gamma_{1\text{-}3}$ heterodimers. Current amplitude after the pre-pulse was normalized to the current amplitude before the pre-pulse. Altogether, TCa$_V$1 currents did not exhibit voltage-dependent inhibition by co-expressed *Trichoplax* G$\beta_1\gamma_{1\text{-}3}$ heterodimers.

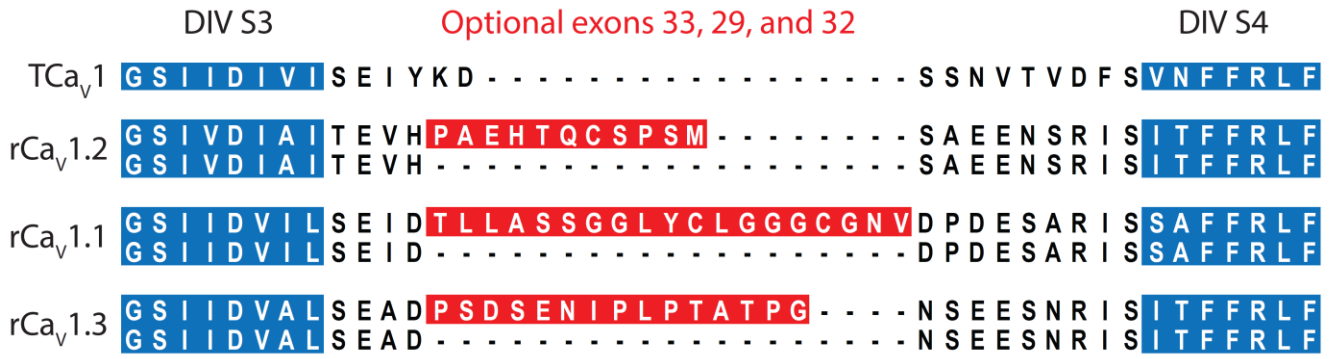

Figure S6. Protein alignment of the DIV S3-S3 loop region of TCa$_V$1 and *Rattus norvegicus* Ca$_V$1 channels, depicting the optional exons 29 (Ca$_V$1.1) (109), 33 (Ca$_V$1.2) (108), and 32 (Ca$_V$1.3) (110).

File S1. Protein sequences and accession numbers of voltage-gated calcium channel protein sequences that were used in this study.

>NP_000060.2|Homo_sapiens_CaV1.1

MEPSSPQDEGLRKKQPKKPVPEILPRPPRALFCLTLENPLRKACISIVEWKPFETIILLTIFANCVALAVYLPMP
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>NP_955630.3|Homo_sapiens_CaV1.2

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GIGKVITISKAIKDGIFEISNSSLCGIEVIGNLPAGCPCNANSDCASKSCYNTSTSSDTRVMQRCGRENVEAVVA
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QPEVMGKLVHKYRIFQRRENIDFQGTCKISDATSSRVVFRQSVNKSWRGFSDDIANQGPFPPFQNDYGCIQDVVG
YEAHPENLGQNRMVYDYFTGPCSEGFFYFIQLPRTNLFLLVVENYLRHDTYFNINCHISRRVTAAGSFELKNGTC
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>XP_004998635.1|Salpingoeca_rosetta_Alpha2Delta/CACHD1

MRAVAAAGLALCTATVVLMVAVDVHGQTTLLNLLQAREQEVEQLATALRGLYDDAYCGSASCASDVAACESELLT
STCASTFGGCRSKTENRRLDFSSSVMRTASTPFDDDVRQEACWTRQLDNTFISINGGTSENTDTATKWQYVGTSS
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YFASLRATTSSPVVWVEPYEDASGAGVLTTASKAVYDTRFSPARLIGVIGIDILASDLQDVAVDYEDLLDALVQR
SNTCPTITLDRCELGAIRTTDYSTSGFPSQYTENDRLCDADELDGCSTTTNLPCVDDNNIYTPPSIPATEETHSH
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>XP_004993504.1|Salpingoeca_rosetta_vWA

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DPSECYAEMNNLLCYFCSPAQSDFYREGVPVVCERMCNRVYRRCKRFSVNGVKMEDAYSSGTKFCEAQGFIVDST
PLADVPRGVPVYSTCHIYLDAASAVHPSIMLLAAVLLVSIGNACGLGSSSESNDEADKAAESKRTRQGTPKYTPA
PTRAPTRSLPSAASTARASASINTTSIMRASVAVAVLALAVLCSVPSATAQTDTVDDADMVAWAAGVAQQLHTMR
SQGTAANHLQNTIDTANYTKETIDAGARARMVADRLSDFFTRKEALARNLAAAAADAYMRHKQNPVILEQYANVD
LPESLPDLTFSPFYKTNVSFAQSGTKVAIDEPQPSQAVDSDIAWTAGLDGDGVFTRNMQNDSDIRWQYVGTERGV
FRQFPARLWDTNFIGFPLDFDPRFRPWYLATLSGPKDIVIVLDCSRSMRGDKWNDAVAMTKFLVNSLSRDDRYNV
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DCPMRDPPRTDCQRLMVFLSDGKDRDNEVRCGRGRYYYTRNGRQYDPPPLCQFKWDDTFDYVRNNAGDIRIFSFG
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ISREHATFKLAAESYCDSVKYINHINDPEYFDNTELFIHTTDTNNTVFEDCPAGFFEMGVVADVRLVQGIEDEWK
GAPSDVIWSYVGTTTGVSLFLPGHRLPHRYDPRTRPWYHQAYSLQNRAVPTIVTSPYLDVSGSGKIITIATTVFQ
SKANITQKACTTYLDCCEVDGDCRLSCYNVDGPCLGSQVGCTCSADSIHSVAAVDVTYETFHQIVEDAVPACTQS
STTRCFVIDSGGLLVYDAEIADALDGSTRSYERISVARRDGSVMRHLVAEGVLTRDDYVDYQGECSSTRPFDRLS
VKGLREGLPPRDADVYDKFRGPQPRHDFLYTCVNDVIAYTAVRDALPVGTVSSGSTQANPCLQSQDMKITPIPNT
NLYLVVAYNRREESTPMPFNCHIFNRAVDSGAFQIVNGTCAAAMTEEEPTLREQEMCPALYPVTLECSFNAASHT
TPPPLVLFSSLLVAVLFLLCN

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