ALLOSTERIC BLOCK OF $K_{Ca}2$ CHANNELS BY APAMIN

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Activation of small conductance calcium activated potassium ($K_{Ca}2$) channels can regulate neuronal firing and synaptic plasticity. They are characterised by their high sensitivity to the bee venom toxin apamin, but the mechanism of block is not understood. For example, apamin binds to both $K_{Ca}2.2$ and $K_{Ca}2.3$ with the same high affinity (K_D ~ 5 pM for both subtypes), but requires significantly higher concentrations to block functional current (IC_{50}'s of ~100 pM and ~5 nM, respectively). This suggests that steps beyond binding are needed for channel block to occur. We have combined patch clamp and binding experiments on cell lines, with molecular modelling and mutagenesis, to gain more insight into the mechanism of action of the toxin. An outer pore histidine residue common to both subtypes was found to be critical for both binding and block by the toxin, but not for block by tetraethylammonium (TEA) ions. These data indicated that apamin blocks $K_{Ca}2$ channels by binding to a site distinct from that used by TEA, supported by finding that the onset of block by apamin was not affected by the presence of TEA. Structural modelling of ligand-channel interaction indicated that TEA binds deep within the channel pore, which contrasted with apamin being modelled to interact with the channel outer pore by utilizing the outer pore histidine residue. This multidisciplinary approach suggested that apamin does not behave as a classical pore blocker, but blocks using an allosteric mechanism that is consistent with observed differences between binding affinity and potency of block.

$K_{Ca}2$ channels (formerly known as SK channels) are characterized by their sensitivity to the highly specific toxin apamin (1). This 18 amino acid peptide, which has been isolated from the honeybee (*Apis Mellifera*) venom (2), contains 2 disulfide bridges that provide a fairly rigid tertiary conformation (3), with two arginine residues (R13 and R14) being critical for its biological activity (4). The cloning of $K_{Ca}2$ channel subunits has revealed the existence of three subtypes ($K_{Ca}2.1$ - $K_{Ca}2.3$, formerly SK1 – SK3) (5) that bind apamin with very high affinity (K_D ~ 5-10 pM) ((6) for a review). However, apamin is less potent at blocking $K_{Ca}2$ current and displays differential block of channel subtypes. For example, $K_{Ca}2.2$ (all species) displays the highest sensitivity, with IC_{50} values from 27 to 140 pM. Rat, human and mouse $K_{Ca}2.3$-mediated currents show an intermediate sensitivity, with IC_{50} values ranging from 0.63 to 19 nM. Finally, human $K_{Ca}2.1$ is the least sensitive, with reported IC_{50} values ranging between 0.7 and 100 nM (6). These differences between binding and electrophysiological results suggest that the mechanism of block by apamin is complex, and that binding and block by the toxin are not identical phenomena.

$K_{Ca}2$ channel subtypes are expressed throughout the CNS and periphery, displaying partially overlapping but distinct locations. This has led to the proposal that block of $K_{Ca}2$ channels may be a novel target for cognitive enhancement, depression and dopamine-related disorders (7). However, blockers would be required to display significant selectivity for particular $K_{Ca}2$ channel subtypes. Differential block of $K_{Ca}2$ subunits by apamin, and even more so by the peptidic blocker Lei-Dab7 (8), has raised considerable interest. It is clear that understanding the

mechanism of this differential block might contribute towards the synthesis of non-peptidic compounds that could selectively target a particular $K_{Ca}2$ subunit (7). In this study, we have used a multidisciplinary approach, including binding, mutagenesis, structural modelling and patch clamp experiments with $K_{Ca}2.2$ and 2.3 channels, in order to gain a comprehensive understanding of how apamin works. Taken together, our results demonstrate that apamin operates with a mechanism that is not consistent with classical pore block.

EXPERIMENTAL PROCEDURES

Cell culture, cell and membrane preparation- Wild-type rat $K_{Ca}2.2$ (GenBank accession no. NM_019314) and human $K_{Ca}2.3$ (GenBank accession no. AF031815) channel DNAs were subcloned into the mammalian plasmid expression vectors pcDNA3 (Invitrogen, Paisley, UK) and pFLAGCMV2 (Sigma, Poole, UK), respectively. Point mutations in $K_{Ca}2.2$ ($K_{Ca}2.2$(H337N),$K_{Ca}2.2$(N337R),$K_{Ca}2.2$(N345G) ,$K_{Ca}2.2$(N368H)) and $K_{Ca}2.3$ ($K_{Ca}2.3$(H522N), $K_{Ca}2.3$(H491N)) were introduced using the Quikchange-XL site directed mutagenesis kit (Stratagene-Agilent, Stockport, UK) and subsequently confirmed by dye termination DNA sequencing.

Channels were transiently expressed in HEK293 cells. For each passage, cells were dissociated using an EDTA solution and maintained in modified essential medium (DMEM) (Gibco, Invitrogen, Paisley, UK), supplemented with 10% foetal calf serum (Gibco, Invitrogen, Paisley, UK) and 1% penicillin/streptomycin (Gibco, Invitrogen) at 37°C. They were plated onto 35 mm dishes (Falcon) 48 hrs before transfection. For electrophysiology, transient transfections of HEK293 cells were made using polyethylenimine (PEI) (Alfa Aesar, Inc., MA, USA), by combining channel plasmid DNA with enhanced green fluorescent protein (EGFP) DNA in ratio of 1:1 to 1:10 (maximal plasmid content 1 µg). Cells expressing EGFP were used for electrophysiology 12-24 hours after transfection.

Membranes were prepared for binding experiments as follows: HEK293 cells were plated on a 100 mm dish for 2 days, then transfected with the corresponding plasmid using the PEI method without EGFP. Cells were harvested after 48 hours with cold PBS (4°C) using 5 ml per dish and centrifuged twice for 10 min at 1000g (4°C). The pellet was resuspended in a lysis buffer (50 mM Tris, BSA 1%, pH 7.4), mixed thoroughly and centrifuged for 10 min at 200g (4°C). The supernatant was centrifuged twice for 20 min at 16000g (4°C) and the pellet was resuspended in another buffer (5 mM Tris, 5.4 mM KCl, pH 8.5), using 1 ml per dish. Protein concentration was determined using a colorimetric protein assay with a bicinchoninic acid kit (Pierce, Rockford, IL, USA). The absorbance was measured at 562 nm with a Multiskan Ascent (Thermo LabSystem, Waltham, MA, USA) spectrophotometer. Glycerol (10%) was then added and aliquots were stored at -80°C.

Electrophysiology-Expressed $K_{Ca}2$ currents recorded in either the whole-cell or excised outside-out patch configurations, were evoked in symmetrical high (~170 mM) K^+ conditions using an internal solution that contained 1 µM free Ca^{2+}. Pipettes were fabricated from KG-33 glass (Friedrich and Dimmock, CT, USA) or from code 1403513 glass (Hilgenberg, Malsfeld, Germany) and filled with an internal solution composed of (mM): KCl (120), HEPES (10), BAPTA (10), Na_2ATP (1.5), $CaCl_2$ (9.65, calculated free $[Ca^{2+}]_i$ 1 µM), $MgCl_2$ (2.34, calculated free $[Mg^{2+}]_i$ 1 mM), pH 7.4 with ~40 mM KOH. Cells were bathed in a control external solution that consisted of KCl (120), HEPES (10), EGTA (10), $CaCl_2$ (6.19, calculated free $[Ca^{2+}]_i$ 60 nM), $MgCl_2$ (1.44, calculated free $[Mg^{2+}]_i$ 1 mM), pH 7.4 with ~40 mM KOH. Some experiments were carried out using a physiological K^+ external solution of composition (mM): NaCl (140), KCl (5), HEPES (10), $CaCl_2$ (2.5), $MgCl_2$ (1.2), D-glucose (10), pH 7.4 with NaOH.

For kinetic experiments, solutions were rapidly exchanged using an RSC200 rapid switcher (Biologic, Claix, France) or a BPS-8 system from ALA Science (ALA Scientific Instruments, Farmingdale, NY, USA).

Apamin, *d*-tubocurarine (*d*-TC) and UCL1684 were purchased from Tocris Biosciences

(Bristol, UK). Apamin and *d*-TC solutions were prepared on the day of experiments from a frozen stock of 100 µM and 1 mM in water, respectively. UCL 1684 (Tocris Biosciences) stock was prepared by dissolving in DMSO to 100 µM and stored in aliquots at -20 ºC. Tetraethylammonium (TEA) (from Sigma, St Louis, MO, USA) solutions were prepared from a 1 M stock solution in water. The reversible K_{Ca} blocker *N*-methyl-laudanosine (NML) (9) was synthesized in-house.

Binding experiments-Saturation binding was carried out in a 10 mM Tris-HCl (pH 7.5) buffer solution containing 5.4 mM KCl and 0.1% BSA. ^{125}I-apamin was obtained from Perkin-Elmer (Zaventem, Belgium), with a specific activity of 81.4 TBq/mmol. Glass fibre filters (Whatman GF/C, Maidstone, Kent, UK) used in these experiments were coated for 1 h in 0.5% polyethylenimine (to prevent apamin from binding to the filter) and then washed with 2.5 ml of the ice-cold buffer just before use. Membrane preparations (final protein concentration: 10 µg/ml) were incubated with increasing concentrations of ^{125}I-apamin for 1h at 0°C. Binding experiments were terminated as follows: aliquots were filtered under reduced pressure through Whatman filters. Filters were rapidly washed twice with 2.5 ml of ice-cold buffer and placed into a vial containing Ecoscint A (7.5 ml) (National Diagnostics USA, Atlanta GA). The radioactivity remaining on the filters was measured using a Packard Tri-Carb 1600TR liquid scintillation analyser with an efficacy of 69%. Non specific binding was determined in parallel experiments in the presence of an excess of unlabeled apamin (0.1 µM) and subtracted from the total binding to obtain the specific binding. Where ^{125}I-apamin binding was not detected with some mutant channels, positive controls were carried out in tandem on wild-type channels. All binding data were obtained from a minimum of two batches of membranes.

Molecular modelling-A homology model of K_{Ca}2.2 was created as previously described (10). Docking was performed using the software suite Sybyl (Tripos, St. Louis, MO, USA). Docking simulations were produced by energy-minimising the ligand using the MMF94s force field in Sybyl (Tripos) and then docking the ligand into the K_{Ca}2.2 tetramer model using the Surflex or FlexX docking modules in Sybyl. Interactions were accepted from the lowest energy binding mode. The NMR solved solution structure of apamin was donated by Dr. D. Wemmer (UC Berkeley).

Data analysis-For saturation binding experiments, data were fit with a Hill equation of the form:

$$Bound/Total\ bound = \frac{[apamin]^{n_h}}{([apamin] + K_D)^{n_h}}$$

with K_D being the dissociation constant of the peptide and n_h the Hill coefficient. Curve fitting was carried out using GraphPad Prism 5.02 (GraphPad Software, San Diego California USA). For all experiments, a 1/Y weighting procedure was used, which gave more weight to the smaller values of radioactivity (i.e. those that are close to the K_D).

For concentration-response relationships in electrophysiological experiments, data points representing current block were fit with a variable slope Hill equation of the form:

$$I/I_{cont} = I_{min}/I_{cont} + \frac{1 - I_{min}/I_{cont}}{1 + 10^{(LogIC_{50} - X) * n_h}}$$

where I_{cont} is the amplitude of current in the absence of drug, I is the amplitude of current observed at a given concentration of blocker ([X], expressed in logarithmic units), IC_{50} is the concentration of blocker which blocks 50 % of sensitive current, and n_h is the Hill coefficient (expressed as negative values, but its absolute value is used in the text). Analysis of kinetic electrophysiological experiments is described in the Results section.

All numerical values are expressed as mean ± S.E.M. Statistical analysis was performed using Prism 5.02 (GraphPad Software, San Diego California USA). Data were analyzed with a parametric or non-parametric test where appropriate.

RESULTS

High affinity binding of apamin to K_{Ca}2.2 and K_{Ca}2.3 channels-The affinity of binding (K_D) of apamin to K_{Ca}2.2 and K_{Ca}2.3 channels was assessed in saturation experiments on membranes prepared from transiently transfected

HEK293 cells (see Methods). Binding of [^{125}I]-apamin was saturable, with K_D values of 7.5 ± 2.3 and 8.4 ± 1.7 pM for $K_{Ca}2.2$ and $K_{Ca}2.3$ respectively (n = 7) (Fig. 1A and B). These values were not significantly different from each other (P = 0.77) and were consistent with previously published values (11). Obtained Hill coefficients were close to unity (0.87 ± 0.03 and 0.8 ± 0.1 respectively), indicating that binding was not co-operative.

Differential block of $K_{Ca}2.2$ and $K_{Ca}2.3$ currents by apamin-Expression of either $K_{Ca}2.2$ or $K_{Ca}2.3$ subunits produced inwardly-rectifying whole–cell currents in the presence of 1 μM intracellular Ca^{2+} (10,12-14) (Fig. 1C and D). Both $K_{Ca}2.2$ and 2.3-mediated currents were blocked by the addition of apamin, but block was incomplete despite addition of a supramaximal concentration of the bee venom toxin (15) (Fig. 1C, D & E). Expressed $K_{Ca}2.2$ current was more sensitive to block by apamin than $K_{Ca}2.3$ current. Fig. 1E shows the fractional block of $K_{Ca}2.2$ and $K_{Ca}2.3$ currents measured at -80 mV in response to increasing concentrations of apamin. The data was fit with the Hill equation, giving for $K_{Ca}2.2$: IC_{50} = 107 ± 31 pM, n_h = 1.8 ± 0.4 (n = 10) and for $K_{Ca}2.3$: IC_{50} = 6.1 ± 1.6 nM, n_h = 1.4 ± 0.1 (n = 12) (6). The fitted Hill slope was significantly greater than unity for $K_{Ca}2.3$ (P<0.02), but not for $K_{Ca}2.2$ (P<0.08), although a trend was observed. These data suggested that some cooperativity might be utilized to achieve block. Finally, block of expressed $K_{Ca}2.2$ and 2.3 current was assessed under physiological K^+ conditions to determine whether the concentration of external K^+ affected the potency of block. $K_{Ca}2.2$ current was blocked by apamin with an IC_{50} = 70 ± 30 pM, n_h = 0.91 ± 0.2 (n = 5), while $K_{Ca}2.3$ was blocked with an IC_{50} = 2.6 ± 0.4 nM, n_h = 1.2 ± 0.1 (n = 9) under physiological conditions. These values were not significantly different from those obtained in symmetrical K^+ conditons ($K_{Ca}2.2$, P = 0.16; $K_{Ca}2.3$, P = 0.25), indicating that the potency of block was not significantly affected by the concentration of external K^+.

A histidine residue in the channel outer pore turret was essential for block by apamin-Particular amino acid residues within the outer pore have been reported to influence the sensitivity of $K_{Ca}2$ channel currents to block by apamin (16). We reported previously that the protonation of an outer pore histidine (H) residue common to both $K_{Ca}2.2$ and 2.3 channels inhibited macroscopic current using an allosteric mechanism (10). It was possible that this H residue may interact with apamin to cause block. The sensitivity to block by apamin of the mutant channels $K_{Ca}2.2$(H337N) and $K_{Ca}2.3$(H491N) were investigated to examine this possibility, in which the common basic H residue had been replaced with an uncharged asparagine (N). Fig. 2A and B shows representative $K_{Ca}2.2$(H337N) and $K_{Ca}2.3$(H491N) currents in symmetrical high K^+ solutions before and after the application of 100 nM apamin. Currents were not significantly blocked by 100 nM apamin (Fig. 2D; $K_{Ca}2.2$(H337N) P=0.22, $K_{Ca}2.3$(H491N) P=0.11, n=5). These data suggested that this common H residue located in the S5-PHelix loop was crucial to the inhibitory interaction of apamin with the channel. Radioligand binding experiments showed no specific binding of [^{125}I]-apamin (up to 300 pM) to either $K_{Ca}2.2$(H337N) or $K_{Ca}2.3$(H491N) channels (n = 3 for both mutants, data not shown). These data indicated that the H residue located in the S5-PHelix loop was critically required for the binding and subsequent block of $K_{Ca}2.2$ and $K_{Ca}2.3$ channels by apamin.

It is possible that the proton acceptor property of the outer pore H residue, rather than the possibility of the residue possessing a net positive charge was required for binding of apamin and subsequent channel block. Mutation of the H337 residue to the positive arginine (R) to yield $K_{Ca}2.2$(H337R) produced a channel current that was also resistant to inhibition by 100 nM apamin (Fig. 2C & D; P=0.43). These data indicated that binding and block by apamin required the electrostatic features of the unionized basic H residue in the channel outer pore.

The H residue in the outer pore turret also contributed to block of $K_{Ca}2.2$ by d-tubocurarine and UCL1684-The structural features of apamin proposed to confer high affinity binding have been used to design organic molecule blockers of $K_{Ca}2$ channels, providing a pharmacophore for $K_{Ca}2$ channel blockers (17,18). A number of small organic molecule blockers of $K_{Ca}2$ channels, including dequalinium, *d*-tubocurarine

(*d*-TC) and several cyclic bis-quinolinium cyclophanes derived from dequalinium (e.g. UCL1684), possess two positively charged quinolinium groups that are spatially separated to be analogous to the separation of the positive guanidinium groups of the two arginine residues in apamin. These organic molecules displace [^{125}I]-apamin binding and are proposed to interact with the apamin binding site (19,20). This would suggest that like block by apamin, block by these organic compounds would also require the outer pore H residue. This was tested by determining the sensitivity of $K_{Ca}2.2$(H337N)-mediated current to block by supramaximal concentrations of UCL1684 (21) (20 nM) and *d*-TC (100 μM) (Fig. 2E). The mutant channel was insensitive to both blockers (Fig. 2E; UCL1684 P= 0.47 n=3, *d*-TC P= 0.12 n=4). These data were consistent with binding displacement studies and indicated that the outer pore H residue in the S5-PHelix loop that is common to all $K_{Ca}2$ channel subtypes was essential to the binding and block by apamin, and organic blockers.

The $K_{Ca}2.2$(H337N) mutant retained sensitivity to TEA-It is possible that the lack of block by apamin, UCL1684 and *d*-TC of $K_{Ca}2.2$(H337N) and $K_{Ca}2.3$(H491N) channel currents resulted from the mutation affecting pore structure. We used tetraethylammonium (TEA) ions to probe for any inner pore structural changes that might have been caused by these mutations. TEA acts as a classic pore blocker, interacting at the extracellular mouth of the selectivity filter in K^+ channels to obstruct K^+ flux (22-25). TEA can therefore be used as a 'molecular caliper' to probe the dimensions of the inner pore under different conditions. Different sensitivities are proposed to indicate differences in pore structure and dynamics (26,27). Fig. 3A&B show current traces from outside-out patches recorded in low external K^+ (to allow iso-osmotic addition of TEA to the bath solution), showing that both WT and mutant $K_{Ca}2.2$ channel currents were blocked by TEA. A plot of the fractional current (I/I_{cont}) measured at – 40 mV against the concentration of TEA was fit with the Hill equation and revealed a sensitivity to block for $K_{Ca}2.2$ of IC_{50} = 1.9 ± 0.24 mM with a Hill slope n_h = 1.1 ± 0.1 and for $K_{Ca}2.2$(H337N), IC_{50} = 7.7 ± 0.6 mM, n_h = 0.9 ± 0.1 (Fig. 3C). These data showed that $K_{Ca}2.2$(H337N) was significantly less sensitive to TEA than WT $K_{Ca}2.2$ ($p<0.001$, n=3). The Hill slopes for both channels were not significantly different from unity ($p>0.05$, n=3), consistent with a one to one binding interaction that would be expected for a pore blocking mechanism (28). The block of $K_{Ca}2.2$(H337N)-mediated current by TEA indicated that the general architecture of the inner pore was maintained by this mutation. However, it is likely that the ~4 fold reduction in sensitivity to TEA suggests that the mutation of the outer pore H residue caused a slight change in the structure of the inner pore mouth where TEA is predicted to bind (see below).

Molecular modelling of apamin and TEA interactions with the $K_{Ca}2.2$ pore region suggested distinct binding sites-Despite significant differences in functional properties, $K_{Ca}2$ channels are likely to share pore architecture with Kv channels (29). The mutation of an outer pore valine (V342) residue in $K_{Ca}2.2$ to the glycine (G) that confers sensitivity of Kv channels to charybdotoxin (CTX), created a CTX-sensitive $K_{Ca}2.2$ channel (29). The similarities in the effect of these mutations on $K_{Ca}2.2$ and Kv channels indicated that both the inner and outer pore architecture are similar in these two channel classes. We used our previously published homology model (10), based on the crystal structure of Kv1.2, to model the interactions of apamin and TEA with the $K_{Ca}2.2$ channel (see Methods for details).

Fig. 4A & B shows the results of a docking simulation of apamin targeted to the S5-PHelix loop region of the $K_{Ca}2.2$ pore region homology model that contains the important H337 residue. Fig. 4A shows a top down view of the channel displayed in ribbon form and the apamin molecule in stick form. The loop regions of the outer pore are indicated by arrows. Apamin was found to interact with a number of the residues in the S5-PHelix loop, specifically forming H-bonds between the toxin residue N2 and channel residue H337, R14 of apamin and channel residues Q339 and Q340, the C1 thiol group of apamin and D338 in the channel outer pore, Q17 residue of the toxin and N345 of the channel and H18 of the toxin and S344 of $K_{Ca}2.2$ (highlighted blue in the pore region sequence displayed). Other residues with electrostatic interactions are highlighted in red. Fig. 4B

shows a transverse view of the interaction. The most striking feature of the docking simulation was the large distance between apamin and the conduction pathway for K^+ ions.

Targeting residues that have been shown to affect TEA sensitivity through mutational studies in $K_{Ca}2$ (23) and Kv channels (30) allowed the interaction between the quaternary ammonium ion and $K_{Ca}2.2$ to be modelled (Fig. 4C and D). Fig. 4C shows a top down view of the channel displayed in ribbon form and the TEA molecule in space filled mode for display, with TEA modelled to interact directly above the selectivity filter (Fig. 4C & D). TEA was modelled to interact within the channel pore by electrostatic interactions with the C=O group of Y362, while the ethyl groups of TEA interact via van der Waals contacts with G363 and D364 within the selectivity sequence, and V366 (highlighted red in the sequence displayed below the docking. These data suggested that apamin does not traverse the pore to cause block by occluding the passage of K^+ ions through the selectivity filter, whereas TEA is likely to act as a pore blocker.

$K_{Ca}2.2$(N345G) displayed a reduced sensitivity to apamin but retained high binding affinity-A mutational approach was used to determine whether the modelled interaction of $K_{Ca}2.2$ and apamin was accurate. The Q17 residue of apamin was modelled to interact with N345 by formation a hydrogen bond. This channel pore residue was mutated to the small uncharged glycine (G) residue to neutralise the polar nature of the wild-type asparagine (N) residue. Fig. 5A displays the concentration response relationship for block of the $K_{Ca}2.2$(N345G) mutant by apamin. The data was fit with the Hill equation and gave values of IC_{50} = 4.5 ± 0.8 nM, n_h = 1.30 ± 0.06 (n = 10), showing that mutation of N345 significantly reduced sensitivity to apamin in comparison with WT $K_{Ca}2.2$ (mean IC_{50}: 107 pM) ($P < 0.001$, Student's t test for unpaired values). The value of the Hill coefficient was significantly larger than 1 ($P < 0.001$, Student's t test). In contrast, TEA sensitivity was not significantly altered by this mutation ($K_{Ca}2.2$ IC_{50} = 2.2 ± 0.3 mM, n_h = 0.97 ± 0.05 (n = 3), $K_{Ca}2.2$(N345G) IC_{50} = 3.8 ± 1 mM, n_h = 0.99 ± 0.09 (n = 4, P=0.84 for IC_{50} and P=0.24 for Hill slope)(Figure 5B), indicating that the inner pore region was unaltered.

Binding affinity of apamin for the $K_{Ca}2.2$(N345G) mutant (K_D 8.9 ± 4.1 pM, n = 4) was not significantly different from binding to WT $K_{Ca}2.2$ channels (P = 0.75, Student's t test for unpaired values, data not shown). The Hill coefficient was 1.01 ± 0.13. These data supported the modelled interaction of apamin and $K_{Ca}2.2$ channel and suggested that the interaction of apamin with N345 was involved in the translation between binding and channel block.

Apamin and TEA blocked $K_{Ca}2$ channels using non-interacting binding sites-The evidence presented so far suggested that apamin may not act as a classic pore blocker as is proposed for TEA. As both interact in the pore region, it was pertinent to further address the question of whether the binding sites for TEA and apamin overlap. Current remaining in the presence of a partially inhibiting concentration of TEA represented a measure of the probability that the channels were not occupied by a TEA molecule ($p(C_{un})$, with possible values between 0 and 1) (31):

$$i/i_{cont} = p(C_{un}) = \frac{1}{1+\frac{[TEA]}{K_{D,app}}}$$

If TEA and apamin were to compete with each other for occupancy of the same binding site such that one could not bind if the other were already bound, the binding kinetics would be described by the following scheme:

$$C{:}TEA \leftrightarrow C_{un} \leftrightarrow C{:}APA$$

In this scheme C_{un} represents the unblocked channel, C:TEA represents the channel bound with TEA and C:APA represents the channel bound with apamin. It is clear that the on-rate for apamin block in the presence of TEA would be dependent on the probability ($p(C_{un})$) of the channel being unoccupied by TEA. The equation for the relaxation time course to equilibrium for a bimolecular reaction following a rapid increase in the concentration of apamin would therefore be modified from:

$\tau_{on} = \frac{1}{k_{-1} + k_{+1}[APA]}$, in the absence of TEA to $\tau_{on} = \frac{1}{k_{-1} + k_{+1}[APA]p(C_{un})}$, in the presence of a constant concentration of TEA. Therefore, the on-rate for apamin should be slower in the presence of TEA if the blocking sites of apamin and TEA overlapped.

The time-course of block by apamin was well fit with a single exponential function. The rate of block by apamin was unchanged when applied after current was partly blocked by application of TEA (Fig. 6A, B & E). The current decay from separate experiments were fit with single exponentials that yielded time constants (τ_{on}) for block by 3 nM apamin of 0.70 ± 0.12 s (n = 6) and 0.79 ± 0.08 s (n = 8) for apamin in the presence of a concentration of TEA that was close to the observed IC_{50} (1.8 mM). In contrast, the rate of block by apamin of current partly blocked by *N*-methyl-laudanosine (NML) was slowed to 1.36 ± 0.08 s in the presence of 5 μM NML and 1.84 ± 0.09 s in 7.5 μM NML (n = 10 and 8, respectively) (Fig. 6C, D & E). The effect of NML was consistent with the $K_{Ca}2$ channel blocker (9) competing with apamin binding (32), while the lack of effect of TEA suggested that TEA and apamin block $K_{Ca}2$ channels by acting at non-interacting and likely separate sites.

Effect of the $K_{Ca}2.2$ N368 position on the sensitivity of the channels to apamin-It was originally reported that N368 within the outer pore region of $K_{Ca}2.2$ was an important contributor to providing a higher sensitivity to block by apamin than observed with $hK_{Ca}2.1$ (16). However, the role of this residue is less clear with the subsequent finding that $hK_{Ca}2.1$ current was not resistant to block by apamin (15). This residue is the only difference between the sequences in the SF-S6 loop region for $K_{Ca}2.2$ (N368) and $K_{Ca}2.3$ (H522) (Fig. 7A) and it is unknown whether this distinction underlies the different sensitivities (IC_{50}) to apamin block of $K_{Ca}2.2$ (~100 pM) and $K_{Ca}2.3$ (~5 nM). Mutation of N368 in $K_{Ca}2.2$ to mimic the pore sequence of $K_{Ca}2.3$ ($K_{Ca}2.2$(N368H)) produced a channel that bound [^{125}I]-apamin with a K_D of 7.1 ± 0.59 pM (n=8), which was not significantly different from binding to WT $K_{Ca}2.2$ (P = 0.85) or $K_{Ca}2.3$ (P = 0.47). In contrast, $K_{Ca}2.2$(N368H) current was blocked by apamin with a sensitivity that was not significantly different from block of WT $K_{Ca}2.3$ (IC_{50} = 2.96 ± 0.6 nM, n_h = 1.47 ± 0.21, n = 7, *P* = 0.09). This value was significantly greater than that observed for WT $K_{Ca}2.2$ (Fig. 7B & D; P< 0.001). These data suggested that N368 was critical for the extra functional sensitivity to block by apamin of $K_{Ca}2.2$ compared with $K_{Ca}2.3$. These findings suggested that $K_{Ca}2.3$ mutated to mimic the pore of $K_{Ca}2.2$ ($K_{Ca}2.3$(H522N)) would display a sensitivity to block by apamin like WT $K_{Ca}2.2$. Perhaps surprisingly, the sensitivity of $K_{Ca}2.3$(H522N) remained unchanged when compared with the WT channel ((P = 0.35, Fig. 7C & D). The data was fit with the Hill equation and gave IC_{50} values of 4.2 ± 0.5 nM, n_h = 1.67 ± 0.12 (n = 8, n_h significantly different from 1, P = 0.0008, Student's t test) (see Discussion).

$K_{Ca}2.2$ channels can be blocked by only one apamin molecule-Co-expression of $K_{Ca}2.2$ and $K_{Ca}2.1$ (16) or $K_{Ca}2.2$ and $K_{Ca}2.3$ subunits (23) produced functional heteromeric channels that displayed sensitivities to blockers that were intermediate to those observed for homomeric channels. We used the same approach using heteromeric channels containing the apamin-sensitive WT $K_{Ca}2.2$ subunits and -insensitive $K_{Ca}2.2$(H337N) subunits to determine how subunit stoichiometry might affect apamin sensitivity. Expressed currents were sensitive to apamin (Fig. 8A). The mean data was best fit by the sum of two Hill equations (Fig. 8B). The high sensitivity component displayed an IC_{50} ($IC_{50,a}$) of 271 pM with a Hill slope ($n_{h,a}$) of 1.3. The lower sensitivity component had an IC_{50} ($IC_{50,b}$) of 33 nM, with an $n_{h,b}$ of 0.85 (n=6). The high sensitivity component made up 62 % of the total block. These data indicated that channels were forming heteromers, displaying two distinct sensitivities to block by apamin. The kinetics of recovery from block of the heteromeric channels confirmed that 2 separate classes of apamin interaction sites existed, with the time-course of recovery being best described by the sum of two exponential functions displaying a fast component, $\tau_{off,\,1}$ = 2.15 ± 0.17 s, and a slower component $\tau_{off,\,2}$ = 56.1 ± 13.5 s (n = 5; Fig. 8C).

The probability of the occurrence (P_{occ}) of different stoichiometries of the tetramer subunit composition can be predicted, assuming that the probability of incorporating either a WT $K_{Ca}2.2$ or a $K_{Ca}2.2$(H337N) channel subunit into the functional channel tetramer during channel assembly was equivalent. Fig. 8D illustrates the possible heteromeric stoichiometries and the P_{occ} of such combinations of subunits. Current derived from homomeric $K_{Ca}2.2$(H337N) channels would not contribute to the inhibition of the macroscopic current, as the current was apamin-insensitive (Fig 2). Therefore, the P_{occ} was adjusted to include current derived only from putatively apamin-sensitive heteromers that included WT subunits. The proportion of channels containing adjacent WT subunits channels gave a P_{occ} value of ~0.6, leaving channels not containing adjacent WT subunits giving a P_{occ} value of ~0.4 (Fig. 8D). Approximately 62% of expressed current was blocked by apamin with a high sensitivity ($frac_a$), leaving approximately 38% of current being blocked by the toxin with a lower sensitivity ($frac_b$)(Fig. 8B). This suggested that apamin must bind to a channel containing at least two adjacent WT subunits to block with high sensitivity. The steep Hill slope (1.3) of the high sensitivity component of block suggested that the positive cooperative binding of more than one molecule of apamin to channels containing adjacent WT subunits provided block of high sensitivity. Therefore, it is likely that current blocked with a low sensitivity represented apamin interacting with channels that contained non-adjacent or only a single WT subunit. This was supported by the lower sensitivity component displaying a Hill slope of 0.85, which suggested no cooperativity of binding exists for apamin blocking these channels. These data suggested that the efficacy of apamin block is influenced positively by interactions between subunits, but that it is also possible for apamin to interact with one sensitive subunit to produce inhibition, albeit at higher concentrations.

DISCUSSION

Point mutations within the outer pore region of $K_{Ca}2$ channels have been shown to affect the sensitivity of block by apamin (16,29), which has led to the assumption that the bee venom toxin acts as a pore blocker. However, it has been recently reported that a point mutation in the S3-S4 extracellular loop had a major impact on the sensitivity of $hK_{Ca}2.1$ current to block by apamin (33). This information places doubt on whether apamin can act as a pore blocker, as it is unlikely that apamin is large enough to bind to an extracellular loop and traverse deep into the pore to cause block.

This study has identified two pore residues that influence apamin sensitivity (H337/H491 of $K_{Ca}2.2$ and $K_{Ca}2.3$ respectively and N345 of $K_{Ca}2.2$) to add to the formerly identified residues (16). Macroscopic current from the mutants $K_{Ca}2.2$(H337N) and the equivalent $K_{Ca}2.3$(H491N) were both insensitive to 100 nM apamin, revealing the importance of the H residue located in the S5-PHelix loop of the outer pore in the apamin interaction. This lack of block arose from a loss of apamin binding. The $K_{Ca}2.2$(H337R) mutant was also insensitive, indicating that it was the proton acceptor property of the H residue that was crucial to both binding and block. We had previously found that external protons allosterically inhibited $K_{Ca}2$ current by interacting with the outer pore H residue (10) and it is possible that positively charged residues on apamin might mimic protons and interact with the H residue. Structural modelling of the interaction of apamin with $K_{Ca}2.2$ channel produced a lowest energy interaction that positioned apamin away from the selectivity filter, interacting with multiple residues in the S5-PHelix loop, including both the outer pore H and some already proposed to mediate the apamin-channel interaction (16). This modelled interaction was supported by the lower sensitivity to block by apamin of the $K_{Ca}2.2$(N345G) mutant. A previous study showing that a serine residue in the S3-S4 loop region contributed to high affinity block of $K_{Ca}2.2$ by apamin suggested that the outer pore region residues alone do not compose the complete binding site, but do so in combination with the S3-S4 loop (33). Based on the orientation of the S3-S4 transmembrane segments within the Kv1.2 structure, it is possible that the S3-S4 loop may come into close contact with the S5-PHelix loop region. Our modelled interaction of apamin with $K_{Ca}2.2$ placed R13 of the toxin projecting away from the pore region of the channel, making it

possible that this residue could interact with residues outside the channel pore. Therefore, our modelled interaction is consistent with mutational studies and suggests that apamin is unlikely to physically occlude the pore.

The $K_{Ca}2.2$(H337N) channel was also found to be insensitive to supramaximal concentrations of $K_{Ca}2.2$ and 2.3 channel organic molecule blockers such as *d*-TC, UCL1684 and NML (data not shown for the latter). These molecules all displace [^{125}I]-apamin binding and must compete for either part or the entire binding site used by the toxin (17-19,32). Therefore, the lack of block by these compounds was not surprising based on the overlap of the binding sites. However, the implications of these data were surprising. It is likely that apamin does not block $K_{Ca}2$-mediated current by occluding the pore. Therefore, it is unlikely that *d*-TC, UCL1684 and NML are pore blockers. However, it is possible that the (H337N) mutation might perturb pore shape that would prevent binding of these organic blockers. We used TEA to investigate whether this might have been the case.

TEA has been used as a molecular caliper to probe the inner pore of Kv channels (27). TEA could be used in the same way for $K_{Ca}2$ channels, but first it had to be determined whether apamin and TEA bound to non-overlapping sites. TEA was modelled to interact close to the selectivity filter of a $K_{Ca}2$ channel, in a region that has been previously proposed based on mutation studies (23). These data suggested that apamin and TEA bound to distinct sites within the pore region of these channels. This was supported by the finding that the presence of TEA did not affect the kinetics of apamin block. In contrast, the kinetics of block was slowed in a concentration-dependent manner by NML, a blocker known to compete for the same binding site as the toxin. These data provided strong evidence that apamin and TEA do not bind to overlapping binding sites. For comparison, an opposite conclusion was drawn concerning TEA and charybdotoxin block of single BK channels (31). It is clear that TEA can also be used with $K_{Ca}2$ channels to probe the channel inner pore. The apamin-insensitive mutant $K_{Ca}2.2$(H337N) was less sensitive to block by TEA (IC_{50}~8 mM) than the WT channel (IC_{50}~2 mM). The reduced sensitivity of $K_{Ca}2.2$(H337N) suggested that the inner pore region was somewhat altered by this mutation, but much less so than the extent to which the apamin site was perturbed. The absolute effect of this mutation on apamin binding indicated that this residue was a significant contributor to the binding site of the toxin. The lack of block of $K_{Ca}2.2$(H337N) by *d*-TC, UCL1684 and NML would suggest that these blockers also bind to the outer pore, a suggestion supported by the fact that these blockers displace apamin binding. Therefore, these compounds are not pore blockers and, like apamin, must inhibit macroscopic $K_{Ca}2$ current by an allosteric mechanism. This suggestion would help to explain the disparity between binding K_D's (~ 8 pM for both $K_{Ca}2.2$ and $K_{Ca}2.3$) and the functional inhibition of current by apamin, particularly in $K_{Ca}2.3$ (IC_{50} ~ 5 nM). This proposed separation of binding and block was supported by the finding that no cooperativity of apamin binding was observed, while positive cooperativity was observed for functional inhibition.

Two residues within the pore sequence of $K_{Ca}2$ channels have been identified that might be involved in translating binding to block. The first was N345 within $K_{Ca}2.2$, where mutation to glycine produced a channel that displayed a lowered sensitivity to block by apamin, but retained high affinity binding for the toxin. The second was N368 within $K_{Ca}2.2$, which corresponds to H522 in $K_{Ca}2.3$. The sensitivity to block by apamin was reduced to that of $K_{Ca}2.3$ by mutation of N368 to H, but high affinity binding for apamin was retained. These data were in accord with previous work, where mutation of the corresponding H in $hK_{Ca}2.1$ to N to mimic the pore sequence of $K_{Ca}2.2$, increased sensitivity to block by apamin (16). The N368 within $K_{Ca}2.2$ is modelled to directly interact with Q340 and not apamin, with Q340 being modelled to interact with the toxin. Therefore, mutation of N368 would be expected to affect block, but not binding of apamin. In contrast, N345 within $K_{Ca}2.2$ is modelled to directly interact with apamin by hydrogen bonding. Mutation of N345 to glycine affected block, but not binding of the toxin. It is clear that at least H337 in the outer pore turret is essential for binding of apamin, while S243 in the

extracellular S3-S4 loop of $K_{Ca}2.2$ is suggested to contribute to high affinity binding (33). Therefore, it is likely that the lack of effect of mutation of N345 on binding of apamin reflects that interaction of the toxin with this residue is crucial in translating binding to block, rather than it significantly contributing to high affinity binding. No residues modelled to interact with apamin will bind K^+ ions, because changes in external K^+ concentration had no effect on the block of either $K_{Ca}2.2$ or $K_{Ca}2.3$ by apamin. Finally, care must be taken when considering apamin binding and block of $K_{Ca}2.3$ current. For example, mutation of $K_{Ca}2.3$ to mimic the $K_{Ca}2.2$ pore ($K_{Ca}2.3$(H522N)), did not have any effect on apamin sensitivity. These data support the suggestion that either the pore shape of $K_{Ca}2.2$ and 2.3 differ (10), or that differences might exist in the mechanism of transduction of binding to block between $K_{Ca}2.2$ and $K_{Ca}2.3$.

The presented modelling and mutagenesis data suggested that apamin binds to the channel outer pore, rather than deep within the inner pore. Therefore, it is possible that multiple apamin molecules bind to the channel simultaneously to cause block. Concentration-response relationships for apamin block of $K_{Ca}2.3$ currents produced Hill slopes that were significantly greater than 1 (with a trend towards this also observed for $K_{Ca}2.2$). This suggested that more than one molecule of apamin binds to cause block and positive cooperativity exists to produce block. The proposal that two or more molecules of apamin bind to produce high sensitivity block was supported by the finding that channel heteromers consisting of the apamin-sensitive WT $K_{Ca}2.2$ and -insenstitive $K_{Ca}2.2$(H337N) subunits displayed two distinct populations of sensitive current. The higher sensitivity site ($IC_{50,a}$ ~270 pM) displayed a Hill slope of >1 and is proposed to correlate with those channels that contained adjacent WT $K_{Ca}2.2$ subunits. In contrast, the lower sensitivity component ($IC_{50,b}$ ~33 nM) displayed a Hill slope of 0.85 and is proposed to comprise current from channels that contained 2 non-adjacent WT $K_{Ca}2.2$ subunits or only one WT $K_{Ca}2.2$ subunit. In contrast, Hill slopes of unity were produced for apamin binding, indicating no positive cooperativity (11,34,35). Clearly, this difference might indicate that although no cooperativity exists between the binding of two or more apamin molecules, adjacent subunits bound with apamin do interact to cause block. In summary, we suggest that apamin does not block $K_{Ca}2$ channels with a simple pore blocking mechanism. It is proposed that apamin binding to the outer pore causes a disruption of the structural coupling between the outer pore region and the selectivity filter, causing collapse of the selectivity filter to impair conduction of K^+ ions. This allosteric hypothesis provides a novel mechanistic basis for block of $K_{Ca}2$ current by apamin, thus aiding the search for subtype-specific non peptidic inhibitors of the $K_{Ca}2$ channel subfamily.

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Footnotes

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Figure Legends

Fig. 1. Binding and block of $K_{Ca}2.2$ and $K_{Ca}2.3$ channel current by apamin. Representative examples of saturation relationships for ^{125}I-apamin binding to expressed $K_{Ca}2.2$ *(A)* or $K_{Ca}2.3$ *(B)* subunits. *C and D,* Whole-cell macroscopic currents derived from ramps from -80 to 80 mV imposed on voltage-clamped HEK293 cells expressing $K_{Ca}2.2$ *(C)* and *(D)* $K_{Ca}2.3$ subunits. Application of increasing concentrations of apamin inhibited macroscopic current. *E,* Concentration-inhibition relationships for apamin inhibition of expressed $K_{Ca}2.2$ and $K_{Ca}2.3$ current.

Fig. 2. H337/H491 residues are critical for block of $K_{Ca}2.2$ and $K_{Ca}2.3$ currents by apamin and organic blockers. *A, B & C,* Outside-out patch (*A & C*) and whole-cell (*B*) macroscopic currents evoked by ramps from -80 to 80 mV in the absence (black trace) and the presence of a supramaximal concentration of apamin (100 nM) (grey trace). Mutation of the outer pore H residue in $K_{Ca}2.2$(H337N) (*A*) and $K_{Ca}2.3$(H491N) (*B*) produced currents that were insensitive to the bee venom toxin. Mutation of H337 in $K_{Ca}2.2$ to the positively charged arginine (H337R) also produced currents that were apamin-insensitive (*C*). *D,* Graph showing the mean ± S.E.M. inhibition by 100 nM apamin of macrosopic current from each mutant. *E,* Graph showing the lack of block of $K_{Ca}2.2$(H337N) by the organic blockers UCL1684 (20 nM) and *d*-TC (100 μM) compared with block of WT $K_{Ca}2.2$- and 2.3-mediated current.

Fig. 3. Block of $K_{Ca}2.2$ and $K_{Ca}2.2$(H337N) channels by extracellular TEA. Representative traces from outside-out patches of expressed $K_{Ca}2.2$ (*A*) and $K_{Ca}2.2$(H337N) (*B*) channel currents evoked by voltage ramps from -100 to 100 mV in the absence and presence of increasing concentrations of extracellular TEA. Experiments were performed in low (5 mM) extracellular [K^+], with increasing concentrations of TEACl being substituted for NaCl. *C,* Concentration-inhibition relationships for block of wild-type $K_{Ca}2.2$ and mutant $K_{Ca}2.2$(H337N) current by TEA (see text for details).

Fig. 4. Structural modelling of the interaction between the $K_{Ca}2.2$ channel and apamin or TEA. *A*, Top-down and (*B*) side view of apamin docked to the outer pore region of $K_{Ca}2.2$. Channel residues discussed in the text are highlighted as follows: H337 (yellow), Q339 (orange), N345 (green) and N368 (cyan). Residues within the channel outer pore and apamin that make contact by hydrogen bonds are coloured blue, while those channel residues making electrostatic interactions are in red. *C & D,* Top-down and side views of the interaction between TEA and $K_{Ca}2.2$. The quaternary ammonium ion is modelled to interact within the inner pore of the channel by electrostatic interactions with the C=O group of Y362 (distance between C=O and N^+ ~ 4 angstroms) and the ethyl groups of TEA interact via van der Waals contacts with G363 (orange), D364 (yellow) and V366 (cyan).

Fig. 5. Reduced sensitivity to block by apamin displayed by $K_{Ca}2.2$(N345G). *A,* Concentration-inhibition relationship for block of expressed $K_{Ca}2.2$(N345G) channel current by apamin. The dashed grey curve shows the sensitivity of block of wild-type $K_{Ca}2.2$ current for comparison. *B,* Concentration-inhibition relationship for block of $K_{Ca}2.2$(N345G) current by extracellular TEA. The relationship of block by TEA of wild-type $K_{Ca}2.2$ current is shown for comparison (dashed grey line), showing that the channel mutation had little effect upon block by the quaternary ion.

Fig. 6. Apamin and TEA block $K_{Ca}2.3$ current by using non-interacting binding sites. *A,* Example trace of whole-cell holding current recorded at -80 mV from a cell expressing $K_{Ca}2.3$ channels and

bathed in high extracellular K^+ solution . Fast application of apamin (3 nM) produced a block with a τ_{on} of 0.70 ± 0.12 s. Fits shown as grey lines in all panels. *B,* Membrane current recorded at -50 mV from a cell expressing $K_{Ca}2.3$ channel subunits. Fast application of TEA (1.8 mM) blocked approximately 30% of current but had no effect on the time-course of subsequent block by fast-applied apamin (3 nM)(τ_{on} of 0.79 ± 0.08 s). *C,* Fast application of NML (5 μM) blocked approximately 40% of expressed $K_{Ca}2.3$ current, with the presence of this channel blocker slowing the rate of block by the subsequent fast application of apamin (τ_{on} slowed to 1.36 ± 0.08 s). *D,* A greater slowing of the rate of block by apamin was produced by a higher concentration of NML, with τ_{on} of block by apamin (3 nM) being slowed to 1.84 ± 0.09 s in 7.5 μM NML. *E,* Graph showing individual values from all experiments of the τ_{on} (Tau) of block by apamin (3 nM) applied in the absence or presence of either TEA (1.8 mM) or NML (5 and 7.5 μM). A one-way ANOVA showed that τ_{on} values were significantly different from each other ($F = 37$, $P < 0.001$). Tukey post-hoc tests showed that the τ_{on} of apamin was unaffected by TEA ($P > 0.05$), but was significantly affected by both concentrations of NML ($P < 0.05$).

Fig. 7. Pore mimicking mutants reveal differences between $K_{Ca}2.2$ and $K_{Ca}2.3$ structure. *A,* Sequence alignment of the pore regions of $K_{Ca}2.2$ and $K_{Ca}2.3$ channels, with residues that differ highlighted in red. *B,* Concentration-inhibition relationships of $K_{Ca}2.2$(N368H), a mutation that produced a $K_{Ca}2.2$ channel whose pore mimicked $K_{Ca}2.3$. This mutation produced a channel current that was blocked by apamin with a sensitivity that was similar to that seen with wild-type $K_{Ca}2.3$ current. The relationships of block by apamin of wild-type $K_{Ca}2.2$ and 2.3 currents are shown for comparison in dashed grey. *C,* Mutation of the outer pore of $K_{Ca}2.3$ to mimic $K_{Ca}2.2$ ($K_{Ca}2.3$(H522N)) produced a current that was blocked by apamin with a sensitivity that was not significantly different from wild-type $K_{Ca}2.3$ current. The relationships of block by apamin of wild-type $K_{Ca}2.2$ and 2.3 currents are shown for comparison in dashed grey. *D,* Bar chart displaying the IC_{50} values showing the reduction in sensitivity in $K_{Ca}2.2$(N368H) compared with WT $K_{Ca}2.2$($p<0.0001$, n=7-10). No significant shift was observed for $K_{Ca}2.3$(H522N) when compared with WT $K_{Ca}2.3$ ($p>0.05$, n=8-12).

Fig. 8. Co-expression of apamin-sensitive wild-type $K_{Ca}2.2$ and apamin-insensitive $K_{Ca}2.2$(H337N) produced heteromeric channels that displayed distinct sensitivities to apamin. *A,* Representative outside-out patch current traces evoked by voltage ramps from -100 to 100 mV, in the absence (con) and presence of increasing concentrations of apamin. *B,* Concentration-inhibition relationship for block of current produced by co-expression of wild-type $K_{Ca}2.2$ and mutant $K_{Ca}2.2$(H337N) channels by apamin. Meaned data was fit with a two component Hill equation, with IC_{50} values of approximately 270 pM and 33 nM, demonstrating that heteromeric channels were expressed. *C,* Example trace of outside-out patch holding current (Vh -80 mV) from a cell co-expressing wild-type and mutant $K_{Ca}2.2$ channels, during the rapid application and removal of apamin (3 nM). Current was blocked by apamin with an exponential time-course (not shown), with the recovery from block being best described by the sum of two exponential components of taus ($\tau_{off,1}$) 1.8s and ($\tau_{off,2}$) 36.4 s. These data suggest that apamin is interacting with two populations of channels that possess different sensitivities to block by the toxin. *D,* The probabilities of occurrence (P_{occ}) of different predicted stoichiometries of channel subunit assembly was calculated assuming that the probability of the inclusion of $K_{Ca}2.2$ and $K_{Ca}2.2$(H337N) subunits into the channel tetramer was equivalent

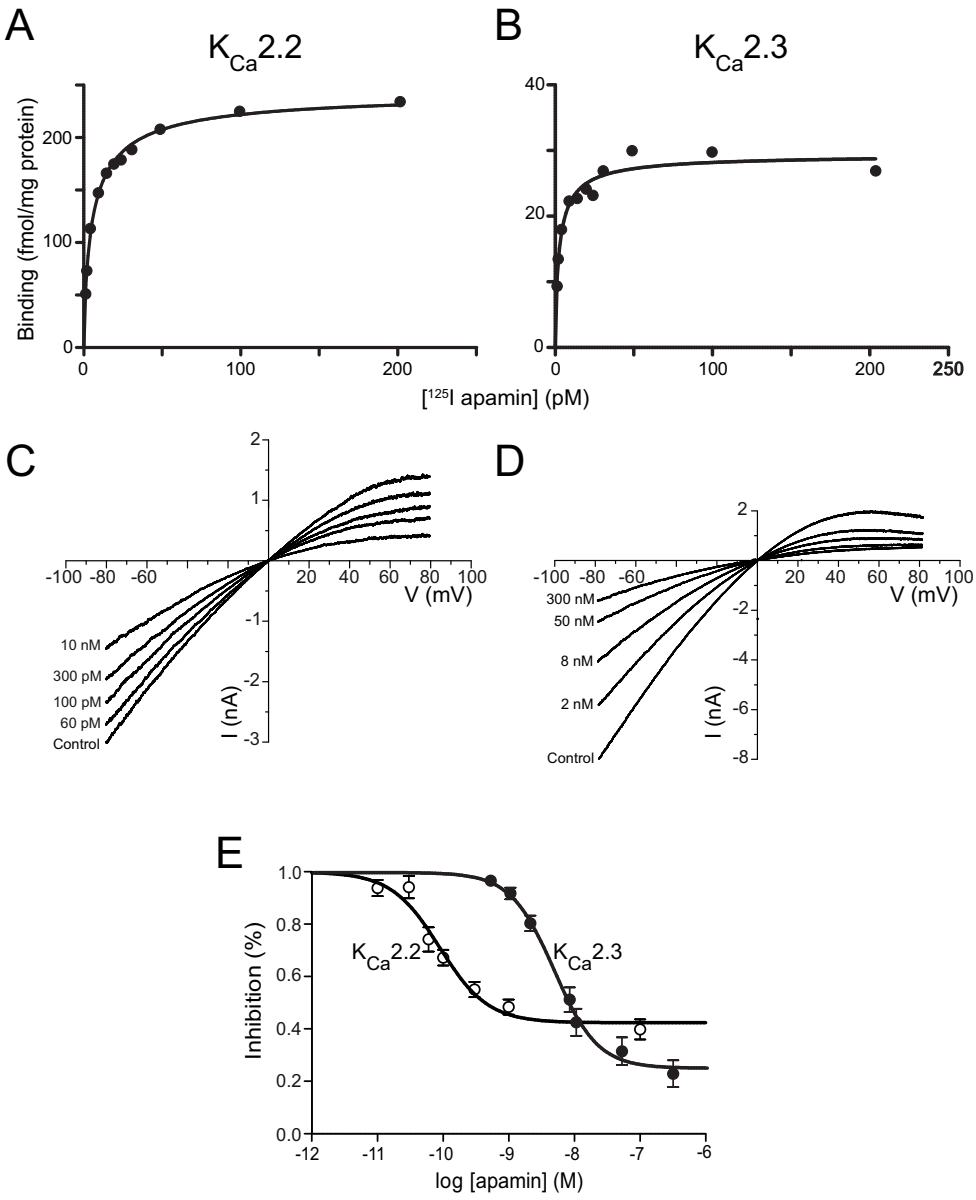


Figure 1 - Lamy et al.

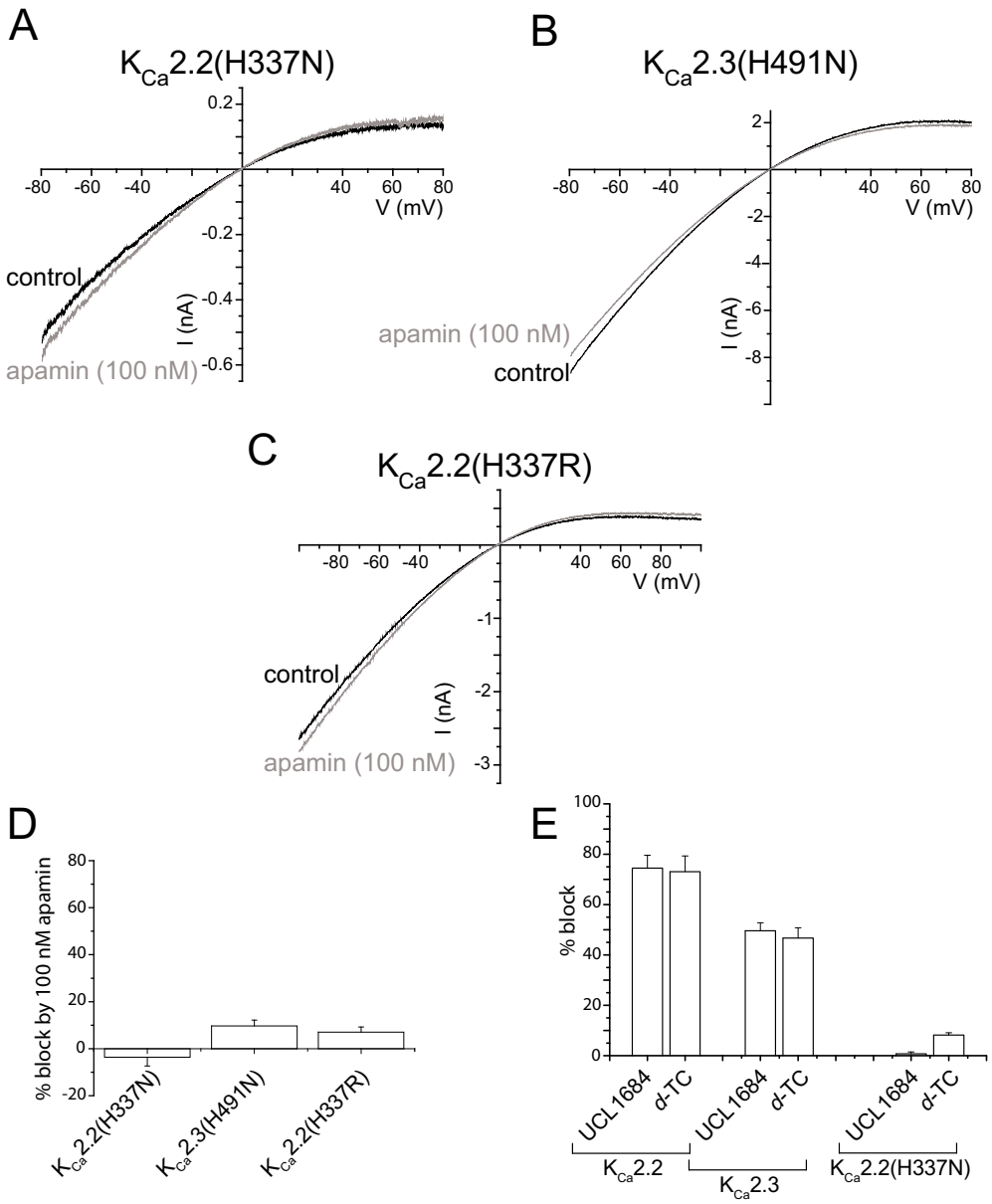


Figure 2 - Lamy et al.

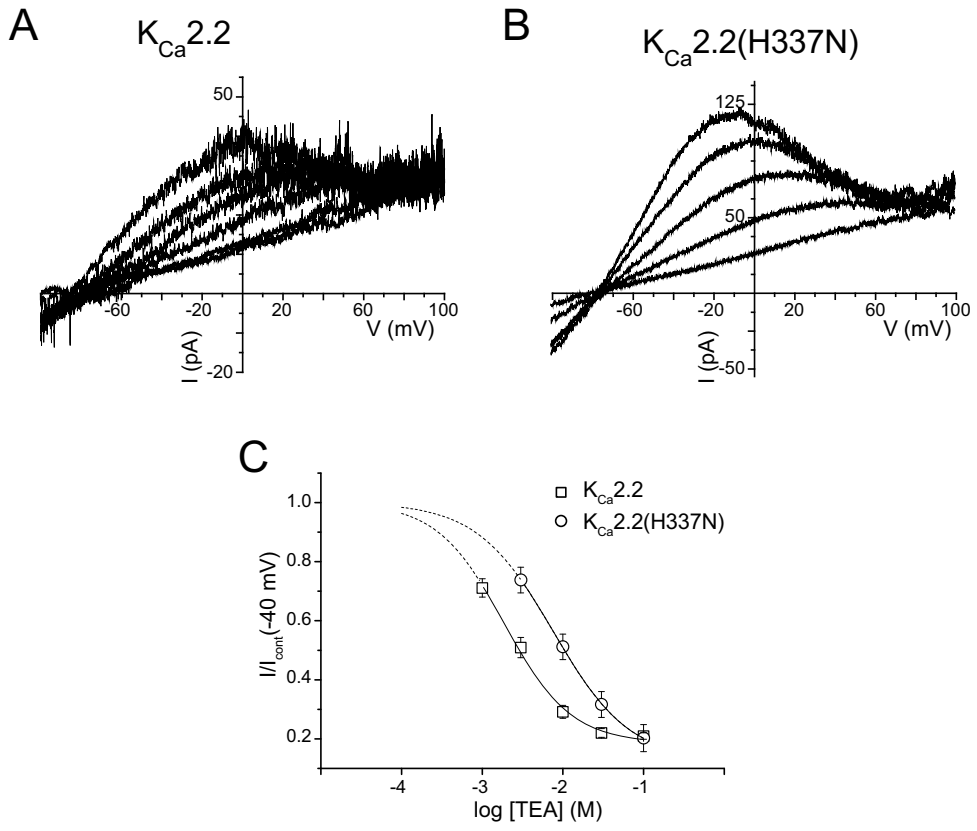
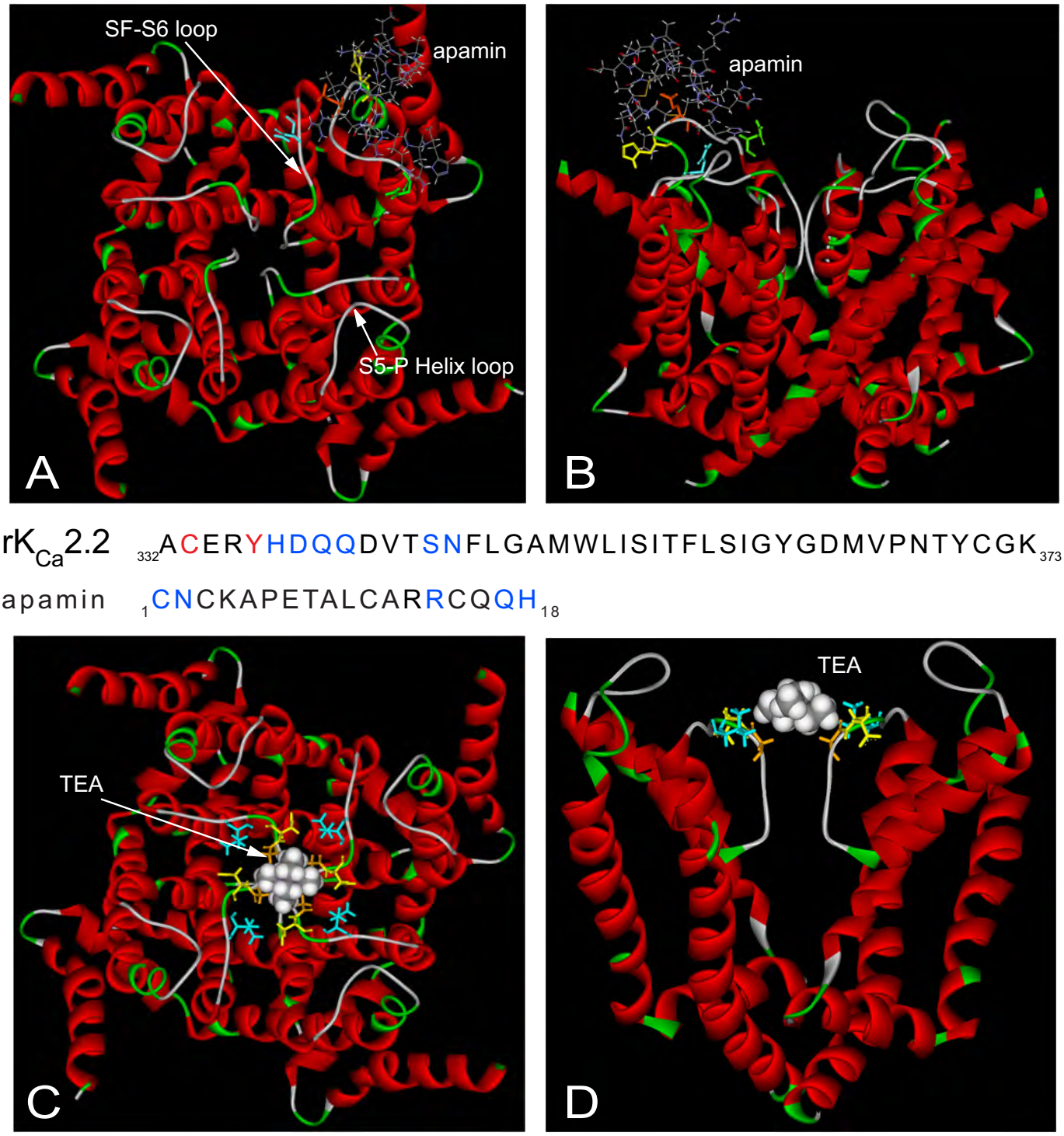


Figure 3 - Lamy et al.



$rK_{Ca}2.2$ $_{332}$ACERYHDQQDVTSNFLGAMWLISITFLSIGYGDMVPNTYCGK$_{373}$

apamin $_{1}$CNCKAPETALCARRCQQH$_{18}$

$rK_{Ca}2.2$ $_{332}$ACERYHDQQDVTSNFLGAMWLISITFLSIGYGDMVPNTYCGK$_{373}$

Figure 4 - Lamy et al.

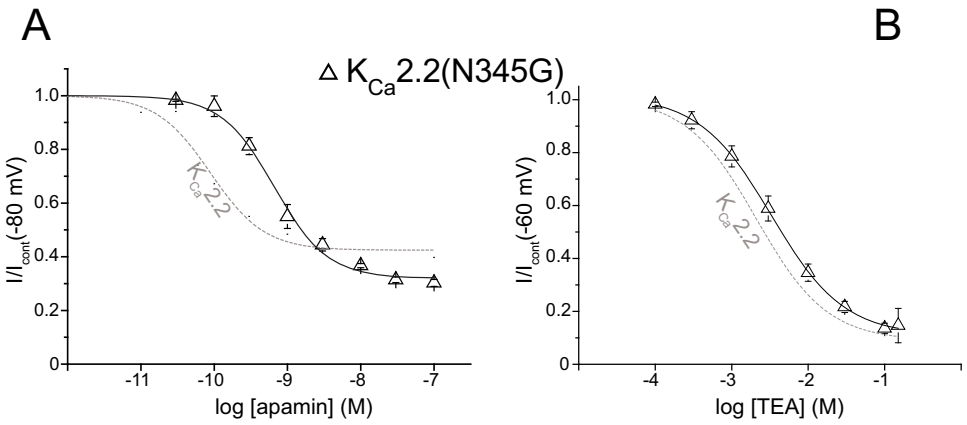


Figure 5 - Lamy et al.

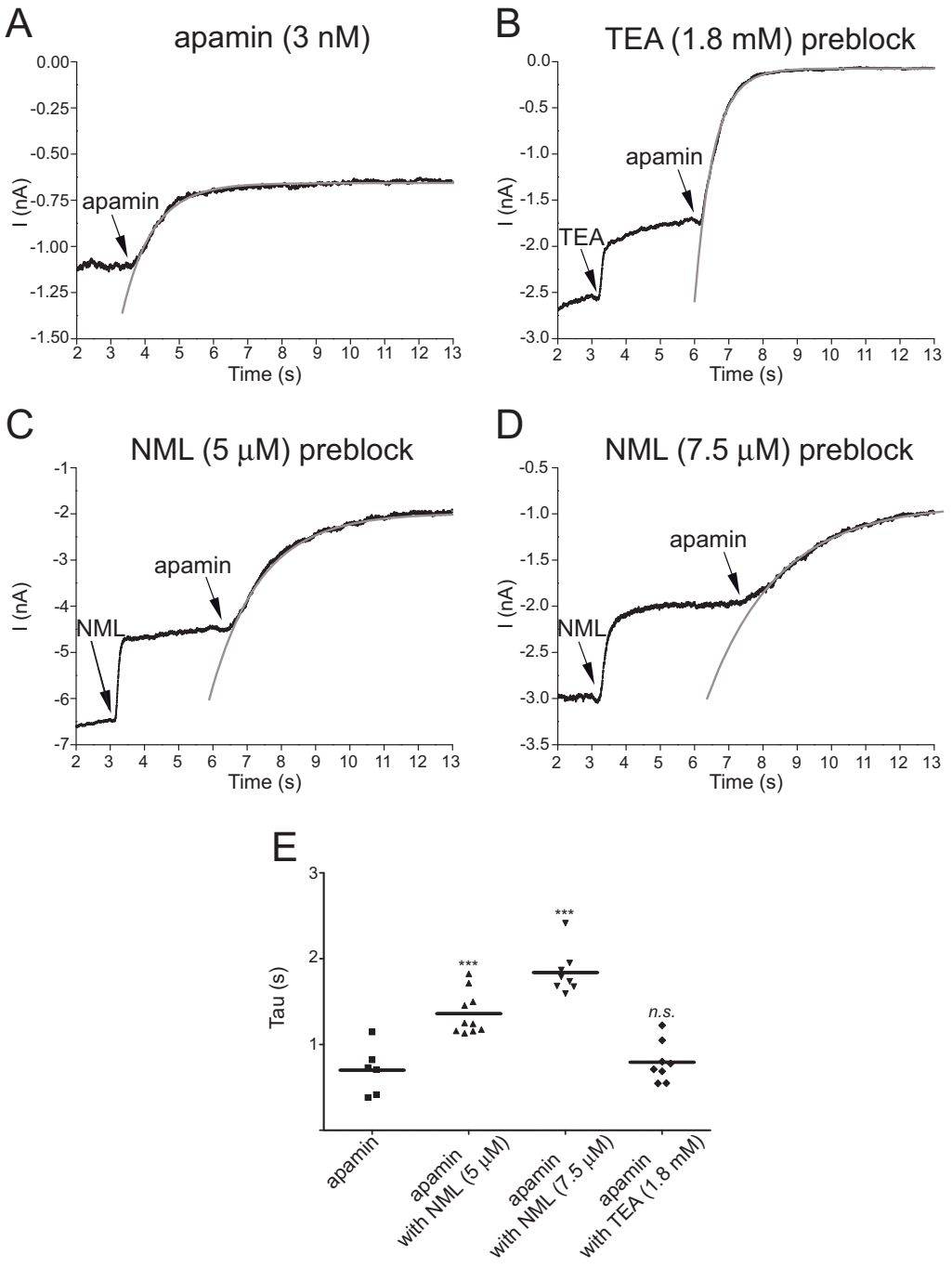


Figure 6 - Lamy et al.

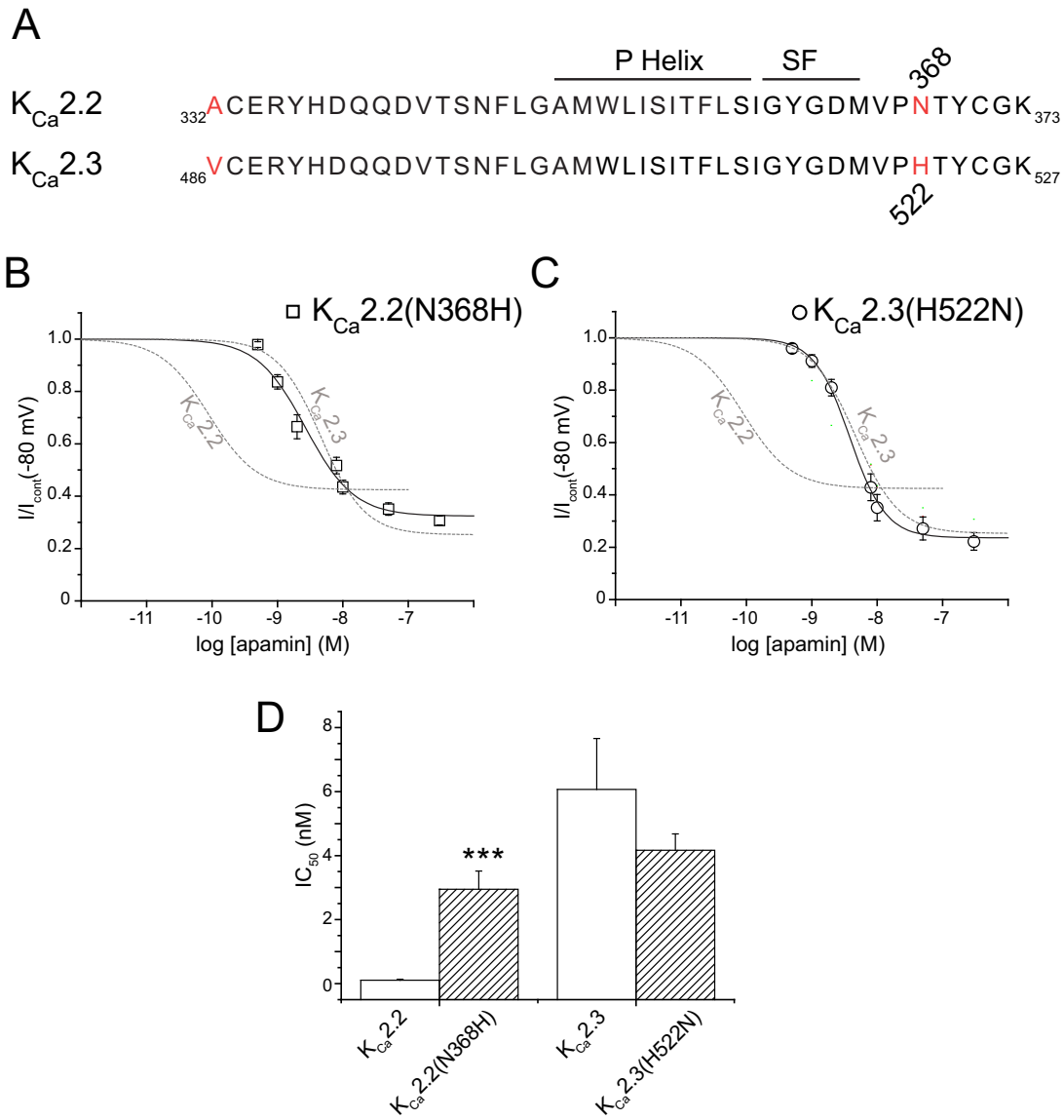


Figure 7 - Lamy et al.

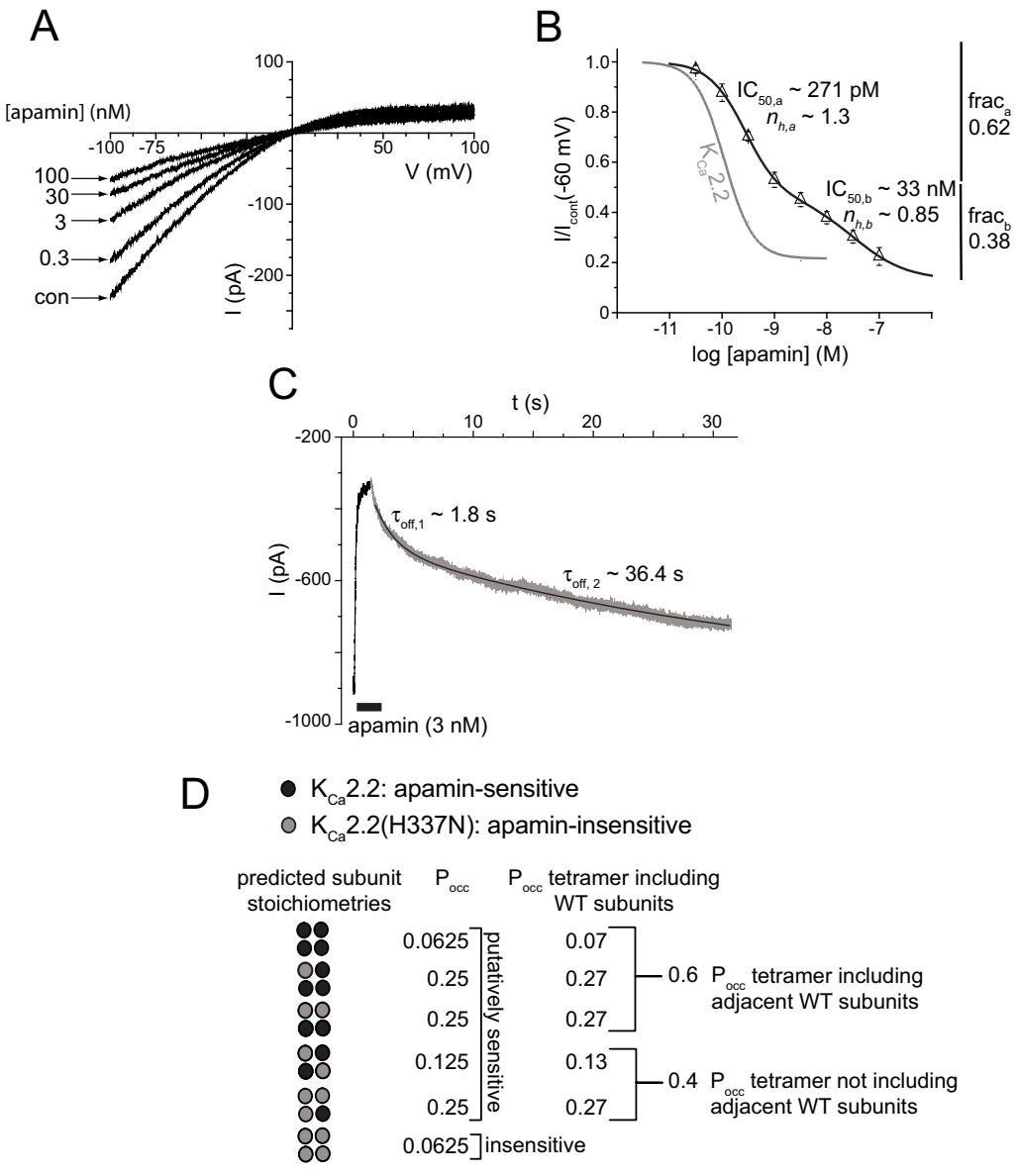


Figure 8 - Goodchild et al.