it remains unclear whether this 3:1 stoichiometry can be extrapolated to all Kv2/KvS channels. Here we report that the silent subunit Kv6.4 can assemble with Kv2.1 subunits into heterotetrameric Kv2.1/Kv6.4 channels with a dimer of dimers configuration. Therefore we constructed both Kv2.1-Kv6.4 and Kv6.4-Kv2.1 dimers. Each dimer produced functional channels characterized by a voltage-dependence of inactivation ($V_{1/2}$ = −59.2 mV and $V_{1/2}$ = −62.9 mV, respectively) that is shifted approximately 40 mV into hyperpolarized direction compared to the voltage-dependence of inactivation of the Kv2.1-Kv2.1 dimer ($V_{1/2}$ = −23.5 mV). These voltage-dependencies are similar to those obtained after expression of Kv2.1 monomers alone ($V_{1/2}$ = −18.2 mV) and after co-expression with Kv6.4 ($V_{1/2}$ = −55.6 mV). In addition, this dimer of dimers configuration was confirmed with Fluorescence Resonance Energy Transfer (FRET) experiments using YFP and CFP tagged dimers. While these data may not rule out an alternate 3:1 stoichiometry, they do indicate that Kv2.1 and Kv6.4 subunits can heterotetramerize into functional channels with a 2:2 stoichiometry.

646-Pos Board B415
K Channel Activation Kinetics
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Based on x-ray analysis and other evidence, the V-gate of a K^+ channel opens after outward movement of the S4 segment of the four subunits, each S4 moving in four steps. K^+ channel kinetics have been empirically described as governed by four "n" particles (Hodgkin and Huxley) each with two possible positions, deactivated and activated, with all four activated for conduction; and more recently as a three state scheme for the S4 segments, resting, preactivated, and activated, followed by an opening 'concerted' step. We examined I_g of nonconducting ShBN W434F. In agreement with previous data, I_g during activation at high voltage has a rising phase, with no rising phase for small depolarizations. Strikingly, the deactivation tails at –80 have only two kinetic components. A fast quasi-exponential tail follows small depolarizations. Following large depolarizations that open all V-gates, the quasi-exponential component is absent, and the initial amplitude of I_g is zero: the open V-gate paralyzes all S4 motion, but I_g rises in amplitude as some V-gates close, then decays as the pool of open gates is exhausted. All I_g tails can be accurately reconstructed from these two components. Fitting with the minimal kinetic scheme (70 states) that describes four S4 segments each with four positions, we find that the 1st step (in which R1 moves from inside, near *Shaker* E293, to outside, near E283) is approximately 10 times slower than the next 3 steps (R2, 3, and 4 move from inside to outside). In the fourth step K5 moves to E293, a relatively stable position. On deactivation when the V-gate is not open, reversal of the fourth step is rate limiting, and the kinetics are quasi-exponential. These findings explain both the kinetics of activation and deactivation, and the steepness of the Q-V curve.

647-Pos Board B416
Activation and Inactivation Steps Altered by Proline Hinge Mutations in Kv1.4
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Several voltage-gated channels share a Proline-Valine-Proline ("proline hinge") sequence motif at the intracellular side of S6. We studied the proline hinge in Kv1.4 channels which inactivate via two mechanisms: N- and C-type. We mutated the second proline to glycine (P558G) or alanine (P558A), and studied these mutations in the presence/absence of the N-terminal to separate the effects of the interaction between the proline hinge and N and C-type inactivation.
Both S6 mutations slowed or removed N- and C-type inactivation, and altered recovery from inactivation. P558G slowed activation and N- and C-type inactivation by nearly an order of magnitude. Sensitivity to extracellular acidosis and intracellular quinidine binding remained, suggesting that the transmembrane communication in N- and C-type inactivation was preserved, consistent with our previous findings of major structural rearrangements involving S6 during C-type inactivation. P558A was very disruptive: activation was slowed more than an order of magnitude, and no inactivation was observed. These results are consistent with our hypothesis that the proline hinge and intracellular S6 movement plays a significant role in inactivation and recovery.

To analyze the effect of these mutations in changing channel gating kinetics, we modified our previously published model of Kv1.4 by adding a primed but non-conducting state connected to the open state by a voltage insensitive step. In order to reproduce the experimental observation that N-type inactivation occurs at more positive potentials than C-type, the C-type inactivated state must be coupled to earlier voltage-dependent steps. Analyses of this model suggest that both P558G and P558A mutations not only modified early voltage dependent steps, but also made the voltage insensitive transition from the primed state to the open state very slow relative to activation.

648-Pos Board B417
Novel Modulation of a Kv1.2 Chimera by Volatile Anesthetics
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Previous work showed that whereas n-alcohols and most general anesthetics at relevant concentrations inhibit the voltage-gated K^+ channel Shaw2 (K-Shaw2), sevoflurane activates it. By contrast, the wild-type Kv1.2 channel is resistant to most general anesthetics except sevoflurane, which also activates this channel. To investigate the structural basis of these differences, we created a new Kv1.2 chimera (Kv1.2-FRAKT). This approach exploits previous findings indicating that the K-Shaw2 S4-S5 linker is a determinant of the channel's modulation by general anesthetics and n-alcohols. We exchanged five residues in the S4-S5 linker of Kv1.2 with residues at equivalent positions in K-Shaw2 (converting QTLKASMRELGLL into QTFRASAKELTLL). Kv1.2-FRAKT was then expressed in Xenopus oocytes and investigated under two-electrode voltage-clamp conditions. Kv1.2-FRAKT exhibits a dramatic rightward shift in the voltage dependence of activation ($\Delta V_{1/2}$ = +41.5 ± 3 mV) and novel responses to general anesthetics. Kv1.2-FRAKT is activated by halothane ($K_{0.5}$=0.47 mM, n_H=1.2), isoflurane, sevoflurane ($K_{0.5}$=1.2 mM, n_H=2.0) and propofol ($K_{0.5}$=20.3 μM, n_H=2.2); however, it remains highly resistant to n-alcohols (1-butanol, $K_{0.5}$=101 mM, n_H=1.5). Surprisingly, complete truncation of the T1 domain abolishes activation of K-Shaw2 by sevoflurane, but has no effect on the generalized activation of Kv1.2-FRAKT by general anesthetics. Therefore, Kv channel regions involved in gating or its regulation play an important role in their differential functional modulation by general anesthetics. We are currently conducting MD simulations and docking calculations to cast more light on the structural basis of the observations. Specifically, by comparing the structural space of docking solutions for the ligand chemotypes against the open and closed membrane-equilibrated structures of Kv1.2, K-Shaw2 and Kv1.2-FRAKT, we expect to identify specific sites potentially related to anesthetic binding and effects. Furthermore, we will search for anesthetic interactions with the isolated T1 domain of each channel. Supported by R01-AA010615 (MC).

649-Pos Board B418
Local Anesthetics on Kv Channels - Closed State Binding of Bupivacaine?
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Local anaesthetics (LAs) are generally assumed to block action potentials by binding to Nav channels, preferentially when in inactivated and/or open state. Recently, it has been suggested that they, in addition or preferentially, bind to Nav channels when in intermediate closed states. This is based on the finding that LAs reduce the peak current more at low voltage steps than at high in voltage clamp experiments.
In previous studies we have concluded that LAs preferentially block Kv channels by binding to exclusively open channels. In the present study we have reanalysed the effect, with special reference to the new findings of closed state binding. We analysed the effects of bupivacaine on Kv3.1 channels expressed in Xenopus oocytes. In contrast to the results from the Na studies, bupivacaine reduced the early current more at higher voltages than at lower. Nevertheless, analysing kinetic models we found that the results are explained by binding preferentially to open channels.
We thus conclude that bupivacaine block K channels mainly in the open state. We also conclude that a time and voltage-dependent block, similar to that reported for Na channels, does not necessarily imply binding of channels in different closed states. Furthermore, the results stress the general fact that a block of the early current in voltage clamp experiments does not necessarily imply that LAs bind to the channel when in closed state, contrary to a widely held view.