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Structure-Activity Relationship of Quaternary Ammonium Ions at the External Tetraethylammonium Binding Site of Cloned Potassium Channels

W. JAROLIMEK, K. V. SOMAN, M. ALAM, and A. M. BROWN[1]

Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas 77030 (W.J., K.V.S., A.M.B.), and Department of Medicinal Chemistry, University of Houston, Houston, Texas 77204-5515 (M.A.)

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SUMMARY

Changes in the chemical structure of the tetraethylammonium (TEA) ion reduce binding affinity at the external TEA receptor of outwardly rectifying potassium channels. To study the mechanism of selective binding, we applied a variety of hydrophilic quaternary ammonium (QA) ions to the noninactivating mutant of Shaker B T449Y, to Kv3.1, and to Kv3.1 mutants, expressed in *Xenopus* oocytes. In outside-out patches, QA ions in which ethyl groups of TEA were replaced by methyl groups had a lower affinity than TEA, whereas changes in binding affinity were minor when propyl groups were substituted for ethyl groups. All channels tested showed this pattern. Changes in free energy of binding correlated well with changes in the computed free energy of hydration of the TEA derivatives that we used. The affinity for TEA derivatives was reduced in Kv3.1Y407T, which is in support of the hypothesis that cation π-electron interaction is involved. Binding affinities of QA ions were higher in Kv3.1 Y407F than in the wild-type, suggesting that the hydroxyl groups of the tyrosines reduce QA binding. The rank order of potency of the QA ions toward the different channels studied was the same. These results indicate that external QA ions bind strongly to hydrophobic π-electron-rich functions. The selectivity, however, is determined by the physical properties of the QA ion.

All K^+ channels that have been studied exhibit a much higher affinity for external TEA than TMA or TPA (1–4). Even a small change in the structure of TEA, such as the substitution of a methyl group for one of the ethyl groups, greatly reduces the binding affinity in frog nerve (3) and molluscan neuron (2).

Outwardly rectifying K^+ channels consist of four α subunits (5), and four tyrosines contribute equally to external TEA binding (1, 6), suggesting that TEA binds within a cage of four aromatic residues by cation π-electron interaction (1). For example, TEA and Me_2Pr_2A are comparable with respect to charge, volume, and surface area but possess binding affinities in Kv3.1 that differ by 2 orders of magnitude (4). Thus, the cation π-electron interaction cannot be responsible for the selective binding of TEA. Recently, we showed that changes in the free energy of binding of QA ions in Kv3.1 wild-type correlated with changes in free energy of hydration (4).

To study the nature of TEA selectivity more thoroughly, we tested a range of TEA derivatives on outside-out patches from *Xenopus* oocytes expressing different K^+ channels. We used the noninactivating mutant of Shaker B (7), in which Thr at position 449 is replaced by a Tyr. It has been shown that this position in Shaker B is crucial for TEA sensitivity: the affinity for TEA increases ~100-fold when aromatic residues (Phe or Tyr) replace Thr (1). We compare the results from Shaker B T449Y with data obtained from Kv3.1 (8) and Kv3.1 with mutations at position 407 corresponding to position 449 in Shaker B. In all channels tested, changes in free energy of binding were greater when methyl rather than propyl groups replaced the ethyl function of TEA. Reduction in the free energy of binding correlated with an increase in the free energy of hydration of the TEA derivatives, and the correlation was similar whether the residue occupying the critical aromatic site was a Tyr (as in the wild-type), Phe, or Thr. The higher QA ion affinity of the Phe mutant compared

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[1] Current affiliation: MetroHealth Center, Cleveland, OH 44109-1998.

ABBREVIATIONS: $\Delta G_{Binding}$, free energy of binding; $\Delta G_{Hydration}$, free energy of hydration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Me_2Pr_2A, dimethyl-dipropylammonium; $BuEt_3A$, butyl-triethylammonium; $MeEt_3A$, methyl-triethylammonium; Me_2Et_2A, dimethyl-diethylammonium; $PrEt_3A$, propyl-triethylammonium; QA, quaternary ammonium; TEA, tetraethylammonium; TMA, tetramethylammonium; TPA, tetrapropylammonium; Et_3A, triethylammonium; Me_3EtA, trimethyl-ethylammonium; Pr_2Et_2A, dipropyl-diethylammonium; $MePrEt_2A$, methyl-propyl-diethylammonium.

with the wild-type indicates that a hydrophobic environment enhances QA ion binding. A similar result could be obtained from quantum mechanical calculations (9). Some of the present results have been published in abstract form (10, 11).

Materials and Methods

Molecular biology and electrophysiology. All experiments were performed using outside-out or cell-attached patches from *Xenopus* oocytes. Site-directed mutagenesis, preparation, and maintenance of the oocytes and RNA injection were performed as previously described (12).

To study the block by QA ions in Shaker B without interference from gating, we used a mutant that did not inactivate during short pulses (7) and in which 40 amino acids in the amino terminus (ΔN 6–46) were removed. We refer to this mutant as Shaker B.

Patch-clamp recordings were performed at room temperature with an Axopatch-1B amplifier (Axon Instruments, Foster City, CA). The composition of the patch pipette solution was 100 mM KCl, 5 mM EGTA and 10 mM HEPES. The bath solution consisted of 100 mM NaCl, 20.5 mM *N*-methyl-D-glucamine, 20.5 mM 2-(*N*-morpholino) ethanesulfonic acid, 4 mM $MgCl_2$, and 10 mM HEPES. The bath solution for tail currents measurement contained 30 or 100 mM KCl, 70 or 0 mM NaCl, 4 mM $MgCl_2$, and 10 mM HEPES. Single-channel currents were measured in the cell-attached mode. The bath solution was the oocyte-intracellular solution that contained 100 mM KCl to zero the resting membrane potential; the patch pipette was filled with 120 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, and 10 mM HEPES. pH in all solutions was 7.3. QA ions were prepared as described previously (4). QA ions were kept in stock solutions and diluted to the final concentration with bath solution. NaCl was replaced by an equal amount of the QA ion for concentrations >10 mM. Solutions were applied by a multibarrel perfusion system, which allows solution exchange in <1 sec (4). The action of QA ions was complete within the response time of the system, so an analysis of kinetics was not attempted. Pulse rate was 0.1–0.2 Hz. Data were filtered 2–5 kHz. The pClamp software (Axon Instruments) was used for generation of voltage pulses and data acquisition (sampling rate, 2 kHz). The P/4 subtraction method was applied to correct for linear leakage and capacitive currents.

For determining dissociation equilibrium constants (K_D), the current amplitude was measured at the end of a 120-msec voltage step to 50 mV from a holding potential of −60 or −90 mV. The effect of a blocker was calculated as the ratio of the current amplitude in the presence of the blocker to the current amplitude during wash (usually identical to the predrug amplitude). For each QA ion, the data from concentration-response measurements were fitted to the equation $I_{QA}/I_o = 1/(1 + [QA]/K_D)$.

The approximately 2-fold higher TEA sensitivity observed in the present outside-out patch experiments in comparison to previously published data (13) may reflect differences in drug application and recording mode.

The Woodhull equation (14) was used to calculate the portion of the electrical field sensed by the QA ion. The valence of all QA ions is +1. In Shaker B T449Y, the voltage dependence was tested between 0 and 90 mV in 10-mV steps when normal bath solution was used. In 30 or 100 mM extracellular K^+, 15-mV steps were applied between 30 and 90 mV for outward currents and between −105 and −45 mV for inward tail currents. The slightly different δ values for TEA in Shaker B T449Y in this study in comparison to a previous study (1) could be due to the different voltage range of the test pulses (0.07, 0–90 mV; 0.04, −10 to +60 mV, respectively).

Free energy computations. The free energy of binding was calculated as $\Delta G_{Binding} = -R \cdot T \cdot \ln(1\ M/K_D)$, where R is the gas constant and T = 298 K. The computation of the free energy of hydration ($\Delta G_{Hydration}$) has been described previously (4). Briefly, $\Delta G_{Hydration}$ of the QA ions was calculated by a continuum solvation model (15), using the modules Solvation and Delphi of InsightII molecular modeling software (Biosym Technologies, San Diego, CA) on a Silicon Graphics Indigo workstation. Parameter set and charges were those of the CFF 91 force field (16). The solute (each QA ion) was first generated and energy-optimized using the Discover module and was assigned a dielectric constant of unity. The solvent was represented as a continuum of dielectric constant 80 (for water). For the calculation of the electrostatic contribution to $\Delta G_{Hydration}$, DelPhi maps the entire system onto a grid and numerically solves the Poisson-Boltzmann equation in its finite difference form. The nonelectrostatic contributions to $\Delta G_{Hydration}$ were calculated as the energy of van der Waals interactions between the solute and the solvent and the entropy penalty for creating a solute-sized cavity in the solvent. $\Delta G_{Hydration}$ is the sum of the electrostatic and nonelectrostatic parts.

Results

The application of QA ions to outside-out patches of Shaker B T449Y resulted in a fast, reversible, and concentration-dependent decrease in the current amplitude (Fig. 1A). As described for Kv3.1 (4), small alterations in the TEA structure molecule resulted in greatly different binding affinities for QA ions in Shaker B T449Y. The order of potency was TEA ≈ $PrEt_3A$ > $MeEt_3A$ > TPA (Fig. 1B). TMA (100 mM) had no effect (four experiments). Thus, the affinities of TMA, TEA, and TPA in our experiments were very similar to previously published data (1).

The difference in binding affinities among various QA ions may be due to the binding mechanism at a single site or might reflect different binding mechanisms at different sites in the K^+ channel. To estimate the location of the binding

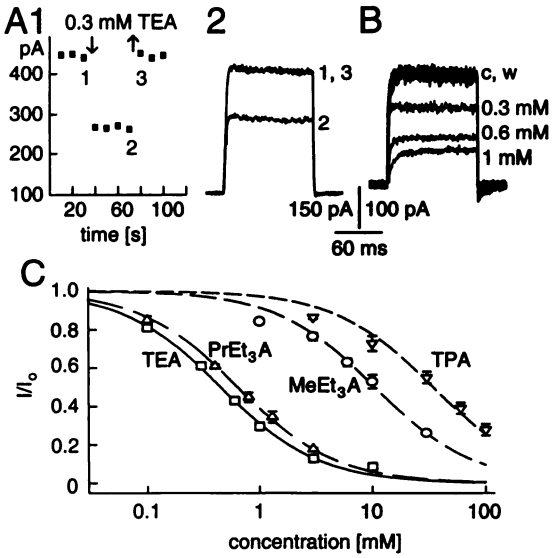

Fig. 1. Effects of QA ions on Shaker B T449Y. A, Application of TEA to an outside-out patch rapidly and reversibly reduced the outward current elicited by a 120-msec pulse from a holding potential of −90 mV to 50 mV. *Arrows*, beginning and end of TEA application. ■, Current amplitude at the end of the test pulse. *Numbers*, traces; shown in full in 2. B, Concentration-dependent reduction of current amplitude. Each drug application was followed by a wash before the next concentration was applied. *c*, *w*, abbreviations for control and wash (all control and wash traces are shown). Activation at high TEA concentrations could be slowed because TEA prolongs bursting due to open-blocked transitions. C, Concentration-response curve for TEA, $PrEt_3A$, $MeEt_3A$, and TPA. *Bars*, standard error, shown if larger than the symbol. *Curves*, fits to the mean values assuming a 1:1 drug binding site stochiometry. Each concentration was tested in 5–12 individual patches.

site, we calculated the fraction of the electrical field (δ) sensed by the blocking ion using the Woodhull equation ((14); see Materials and Methods). As shown in Fig. 2, $MeEt_3A$ reduced inward and outward currents in a voltage-dependent manner. The decrease in binding affinity with increasing depolarization is consistent with block at an external site at which a small portion of the transmembrane field is sensed. The δ values of QA ions varied considerably. Me_3EtA, the smallest QA ion that distinctly reduced K^+ currents in Shaker B T449Y, displayed a strong voltage-dependent block (Table 1), indicating that it binds deeper in the channel than all other QA ions. Other QA ions, with the exception of TPA, had δ values between 0.05 and 0.15. However, there were no systematic changes in δ values with any geometric parameter (Table 1).

The small differences in δ values could indicate that these QA ions bind at slightly different levels within the cage of four Tyr. Therefore, the binding mechanism appears to determine QA ion selectivity. To find a parameter that could account for the different binding affinities, we explored the possibility that K_D may be related to the volume (Fig. 3A) or the surface area (Fig. 3B) of QA ions. However, our results do not reveal any correlation.

As previously shown for Kv3.1 (4), the rank order of K_D values in Shaker B T449Y for QA ions having a similar size to TEA revealed that shortening of one ethyl group to methyl in the QA ion reduced the binding affinity significantly, regardless of volume or size. An even larger reduction was observed when two ethyl-to-methyl substitutions were made (Fig. 3, A and B). On the other hand, lengthening one or two ethyl groups to propyl did not greatly alter the binding affinity. In Kv3.1, these effects could be explained by a mechanism in which the binding of the QA ion includes partial dehydration, and the change in the free energy of hydration

TABLE 1

Calculated dimensions and hydration energies for QA ions and S values for block of Shaker T449Y.

Number of patches is given in parentheses.

QA Ion	Surface	Volume	$\Delta G_{Hydration}$	δ
	Å²	Å³	kcal/mol	
TMA	129	78	−53.36	[a]
Me_3EtA	147	92	−51.95	0.35 ± 0.02 (7)[b]
Me_2Et_2A	165	106	−50.51	0.15 ± 0.01 (11)[c]
Et_3A	167	108	−53.29	[a]
$MeEt_3A$	183	120	−48.97	0.14 ± 0.01 (11)[c]
TEA	199	130	−47.47	0.07 ± 0.01 (13)
$MePrEt_2A$	201	134	−48.84	0.10 ± 0.02 (6)
Me_2Pr_2A	206	138	−49.50	0.14 ± 0.02 (5)[c]
$PrEt_3A$	219	145	−47.00	0.07 ± 0.01 (6)
Pr_2Et_2A	238	160	−47.11	0.05 ± 0.01 (5)
$BuEt_3a$	238	168	−46.98	0.05 ± 0.02 (6)
TPA	278	190	−45.57	0.00 ± 0.02 (7)[c]

[a] Not determined.
[b] $p < 0.001$ (Student's t test) compared with TEA.
[c] $p < 0.01$ (Student's t test) compared with TEA.

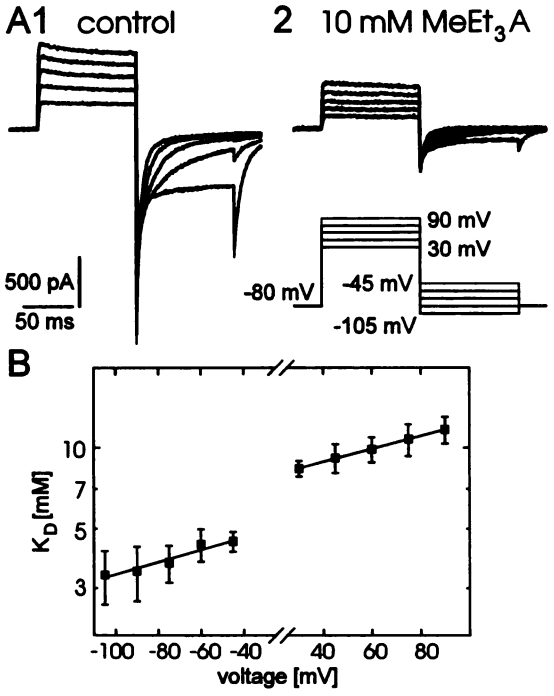

Fig. 2. Voltage dependence of the external block of Shaker B T449Y by $MeEt_3A$. A, Family of currents elicited by voltage steps from a holding potential of −80 to +30 mV for 100 msec and back to −45 mV for 100 msec. *Inset*, depolarizing and hyperpolarizing voltages were increased by 15 mV. A1, Outside-out patch-clamp recording in symmetrical (100 mM) potassium. A2, $MeEt_3A$ (10 mM) reduced inward tail currents to a greater extent than outward currents. B, Plot of K_D values against the test potential. Linear regression of K_D values calculated from the reduction of inward and outward currents yielded lines with the same slope (0.136 and 0.138 for the negative and positive voltage range). Note the gap in the voltage axis. ■, Mean of 12 patches in which four different concentrations were tested. *Bars*, standard error.

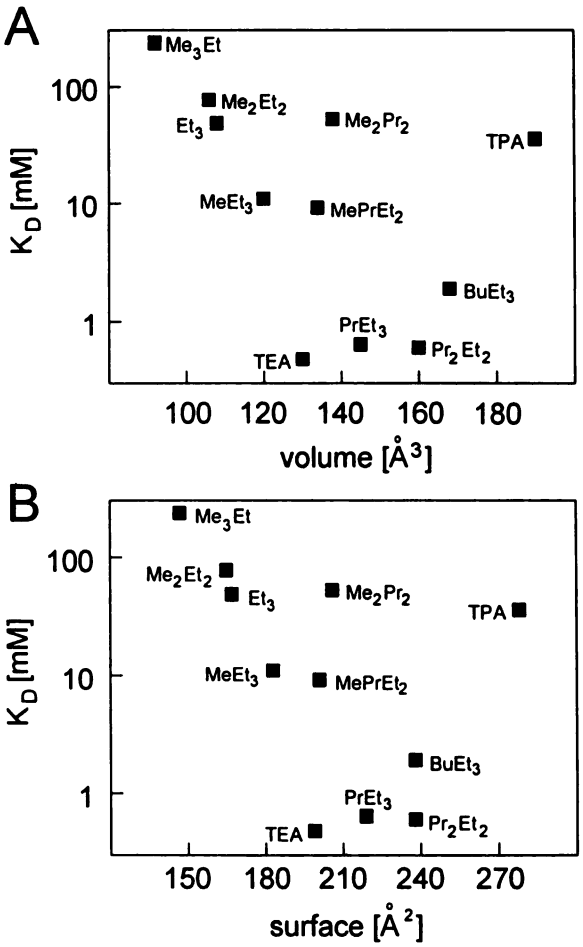

Fig. 3. Plots of the K_D values of QA binding to Shaker B T449Y channel against their volumes (A) and surface areas (B).

($\Delta\Delta G_{Hydration}$) correlated with a change in the free energy of binding ($\Delta\Delta G_{Binding}$, (4)). $\Delta\Delta G_{Hydration}$ of the QA ions correlated with $\Delta\Delta G_{Binding}$ (r = 0.975; Fig. 4A) to Shaker B T449Y. All QA ions were also tested with Kv3.1, and $\Delta\Delta G_{Hydration}$ correlated with $\Delta\Delta G_{Binding}$ (Fig. 4B), as previously described for some QA ions (4). If a similar binding mechanism for TEA derivatives exist to Shaker B T449Y and Kv3.1, $\Delta\Delta G_{Binding}$ values for QA ions in both channels should be related. As shown in Fig. 4C, $\Delta\Delta G_{Binding}$ values for QA ions in Shaker B T449Y and Kv3.1 are highly correlated (r = 0.985).

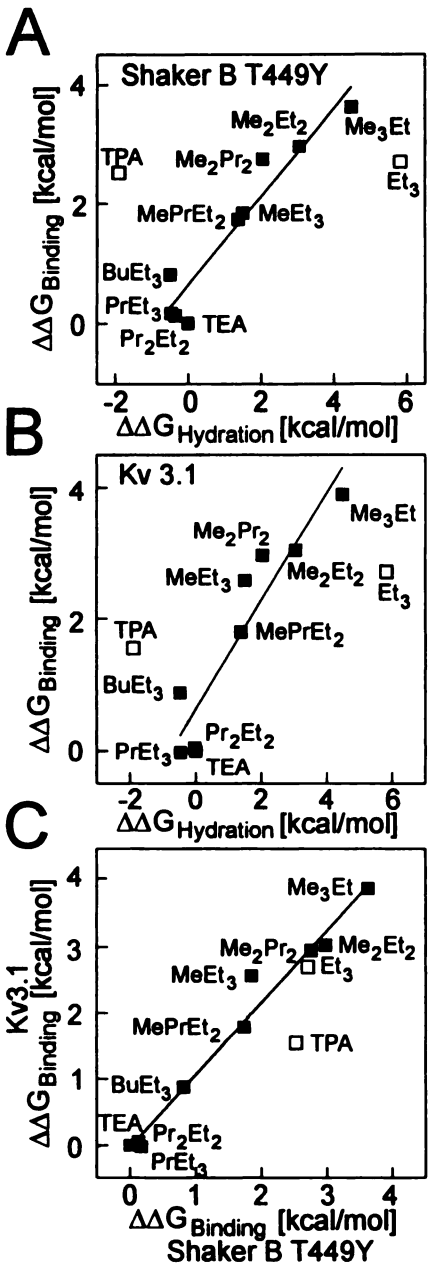

Fig. 4. Correlation of changes in the free energy of QA ion binding ($\Delta\Delta G_{Binding}$) to Shaker B T449Y and Kv3.1 channels with changes in the free energy of hydration ($\Delta\Delta G_{Hydration}$). A, $\Delta\Delta G_{Binding}$ values for Shaker B T449Y were calculated as described in Materials and Methods. A positive difference in $\Delta\Delta G_{Hydration}$ signifies stronger hydration, whereas a positive difference in $\Delta\Delta G_{Binding}$ indicates a reduced affinity. □, Ions that were not included in the correlation. B, Correlation of $\Delta\Delta G_{Binding}$ and $\Delta\Delta G_{Hydration}$ in Kv3.1 for the same QA ions tested in A. C, Correlation of $\Delta\Delta G_{Binding}$ for QA ions to Shaker B T449Y versus that for Kv3.1. *Solid line*, regression line in all three plots.

Although there was a high correlation between $\Delta\Delta G_{Hydration}$ and $\Delta\Delta G_{Binding}$ for most of the QA ions, these values were not related for TPA and Et_3A in the case of Shaker B T449Y and Kv3.1. As discussed below, the TPA binding site has different properties than the TEA binding site. Et_3A is a protonated tertiary amine and not a QA ion. Thus, it is unlikely that $\Delta\Delta G_{Binding}$ correlates with $\Delta\Delta G_{Hydration}$. However, assuming a similar binding mechanism for QA ions similar in size to TEA, we would expect $\Delta\Delta G_{Binding}$ values for Et_3A in Shaker B T449Y and Kv3.1 to be similar, and that occurred (Fig. 4B, Table 2).

The high correlation between $\Delta\Delta G_{Hydration}$ and $\Delta\Delta G_{Binding}$ values for QA ions to Kv3.1 and Shaker B T449Y indicates that hydration energy determines whether a QA ion of similar size to TEA has high or low affinity at the extracellular TEA binding site. Kv3.1 and Shaker B T449Y have a Tyr at the critical TEA binding site of delayed rectifying K^+ channels (1, 13). To define the function of the Tyr residue for QA ion selectivity, Kv3.1 was mutated at position 407, which corresponds to position 449 in Shaker B. To exclude effects related to different gating properties of the mutants, we determined the voltage dependence of activation. As shown in Fig. 5, the mutations did not affect the activation properties. The single-channel conductance for K^+ was slightly reduced (Kv3.1, 22.6 ± 0.9 pS, five experiments; Kv3.1 Y407T, 18.8 ± 0.8 pS, four experiments; Kv3.1 Y407F, 15.7 ± 1.1 pS, five experiments), and the Rb^+ conductance was greatly reduced in the Thr mutant but unchanged in the Phe mutant (Kv3.1, 15.8 ± 0.7 pS, four experiments; Kv3.1 Y407T, 6.9 ± 0.6 pS, five experiments; Kv3.1 Y407F, 15.2 ± 0.7 pS, five experiments). In agreement with these results, K^+ conductance of Shaker B wild-type and T449Y mutant are similar (1, 12), whereas Rb^+ conductance differs (12).

Substituting Thr for a Tyr in Kv3.1 resulted in a large reduction in TEA binding affinity as has been previously shown for Shaker B (1) (Table 2). In agreement with the reduction in TEA affinity, the binding affinity for all other QA ions tested also decreased. No QA ion bound with a higher affinity than TEA. The rank order of potency of the QA ions for Kv3.1 Y407T was similar to those of the wild-type, i.e., $MeEt_3A \ll PrEt_3A \sim TEA$. Due to the generally reduced potency of QA ions for the Thr mutant, some QA ions were ineffective. For QA ions that had an effect, $\Delta\Delta G_{Binding}$ and $\Delta\Delta G_{Hydration}$ were again correlated for Kv3.1 Y407T (Fig. 6A). Furthermore, $\Delta G_{Binding}$ values in Kv3.1 wild-type and Kv3.1 T407Y were linearly related (Fig. 6C, r = 0.99) indicating that the rank order of selectivity was unchanged in the mutant. Interestingly, TPA binding was eliminated by changing the Tyr to a Thr, whereas the same mutation in Shaker B resulted only in a 3-fold reduction in binding (Table 2; Shaker B wild-type, 150 ± 10 mM, 15 experiments).

Aromatic groups at this position are important for the high affinity binding of TEA, and it has been shown that the binding affinity increases when Tyr is replaced by Phe (1, 17). We tested whether $\Delta\Delta G_{Binding}$ for the QA ions in Kv3.1 Y407F were correlated with $\Delta\Delta G_{Hydration}$. In agreement with data from Shaker B (1) and Kv1.1 (17), TEA affinity is higher in the Phe mutant than in the Tyr mutant. The $\Delta\Delta G_{Binding}$ values for the Kv3.1 Y407F mutant again correlated with the $\Delta\Delta G_{Hydration}$ values (r = 0.980, Fig. 6B). Changes in binding

TABLE 2

Binding affinities of QA ions for different K^+ channels

All K_D values are expressed as mean ± standard error. Data were calculated assuming a 1:1 binding site. Each QA ion was tested in at least four different concentrations, with each concentration in at least five patches. Kv3.1 Y407 F had significantly ($p < 0.01$) smaller K_D values than Kv3.1, except for Pr_2Et_2A and TPA.

QA ion	K_D			
	ShakerB T449Y	Kv3.1	Kv3.1 Y407F	Kv3.1 Y407T
		mM		
TMA	234 ± 25	65 ± 4.1	45 ± 4.0	>150
Me_2Et_2A	78 ± 5.5	15 ± 1.1	10 ± 0.55	>150
Et_3A	49 ± 2.6	8.5 ± 1.5	n.d.	n.d.
$MeEt_3A$	11 ± 0.8	6.8 ± 0.9	2.0 ± 0.11	150 ± 30
TEA	0.48 ± 0.02	0.085 ± 0.002	0.056 ± 0.002	7.3 ± 0.6
$MePrEt_2A$	9.2 ± 1.1	1.8 ± 0.21	1.3 ± 0.11	63 ± 15
Me_2Pr_2A	53 ± 2.2	13 ± 1.1	9 ± 1.2	>150
$PrEt_3A$	0.64 ± 0.02	0.082 ± 0.004	0.057 ± 0.003	9.1 ± 0.7
Pr_2Et_2A	0.60 ± 0.03	0.093 ± 0.003	0.085 ± 0.002	12 ± 0.9
$BuEt_3A$	1.90 ± 0.15	0.38 ± 0.03	0.22 ± 0.01	18 ± 0.2
TPA	36 ± 2.6	1.2 ± 0.01	2.4 ± 0.2	>150

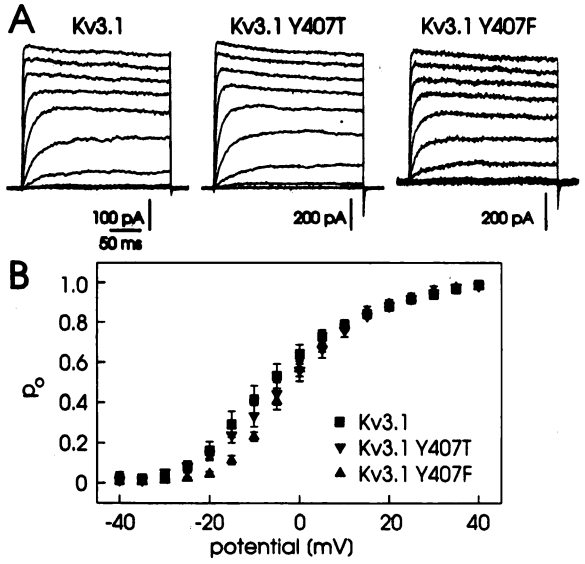

Fig. 5. Effect of the Kv3.1, Y407T, and Y407F mutations on voltage-dependent activation. A, Cell-attached macropatch recordings from Kv3.1 wild-type, Kv3.1 Y407T, and Y407F. Bath solution contained 100 mM K^+, and pipette solution contained 2.5 mM K^+. Patches were held at −80 mV and stepped for 250 msec to potentials from −40 to +40 mV in 5-mV increments. For clarity, only every second current response is shown. B, Voltage dependence of activation calculated from the current at the end of the depolarizing voltage step shown in A. Symbols represent mean of five to seven patches. *Bars*, standard error.

affinity for Kv3.1 Y407F correlated with changes in binding affinity in Kv3.1 wild-type, indicating that the rank order of potency is similar in all mutations (Fig. 6C). Again, binding of TPA is different from the binding of TEA derivatives in that its affinity is higher in Kv3.1 than in Kv3.1 Y407F (Table 2).

Discussion

We found that changes in the free energy of binding of TEA derivatives correlate with changes in their hydration energy. This finding suggests a general mechanism of QA ion block by which QA ions must be at least partially dehydrated to bind to the extracellular TEA binding site of delayed rectifying K^+ channels. By mutating a critical residue for external TEA binding in Kv3.1, we found that the potency of TEA binding depended on which amino acid was present at that position, with the order of potencies being Phe > Tyr ≫ Thr. The QA selectivity, however, did not depend on the binding site but was mainly determined by the free energy of hydration of the QA ion.

Structural features of QA ions at the TEA binding site. QA ions in which one or two ethyl groups were replaced by methyl or propyl groups were used to investigate the binding mechanism at the external TEA receptor in delayed rectifying K^+ channels. TEA derivatives that have a similar volume and surface area have affinities that differ by as much as 100-fold for Shaker B T449Y, Kv3.1 wild-type, and Kv3.1 mutants. A similar selectivity has been found in *Aplysia* pacemaker neurons and in the frog node of Ranvier (2, 3).

With the exception of Me_3EtA in Shaker B T449Y and TMA in Kv3.1(4), all QA ions displayed similar voltage-dependent binding, indicating that they act at the same locus. The TEA binding site can accommodate QA ions with a volume ranging from 106 $Å^3$ (Me_2Et_2A) to 160 $Å^3$ (Pr_2Et_2A corresponding to a diameter of ~8–9 Å (8 Å has been suggested previously (1)). In accordance with the idea that QA ions bind within a cage of four Tyr side chains (1, 6), bulky QA ions appear to be favored over QA ions with a more extended structure. For example, Pr_2Et_2A has a higher binding affinity than $BuEt_3A$, which has almost the same surface area, volume, and hydration energy.

Our results suggest that TPA and Me_3EtA do not bind to the TEA binding site in Shaker B T449Y. In view of the strong voltage dependence of block, Me_3EtA appears to bind deeper in the pore than TEA. The different voltage dependence of block does not, however, result from a different mechanism of block. Free energies of binding for Me_3EtA correlated with free energies of hydration in Kv3.1 wild-type, Kv3.1 Y407F, and Shaker B T449Y. Clearly, QA ions must also be partially dehydrated to bind deeper in the pore. There are several lines of evidence suggesting that TPA does not bind with the same binding mechanism as TEA. The binding of TEA but not of TPA to Kv2.1 is voltage dependent (4). Furthermore, mutations at a crucial residue in Shaker B and Kv3.1 changed K_D values for TEA, but these changes were unrelated to changes in the K_D values for TPA. Most probably, the larger diameter and lower surface charge density of TPA result in a different binding mechanism.

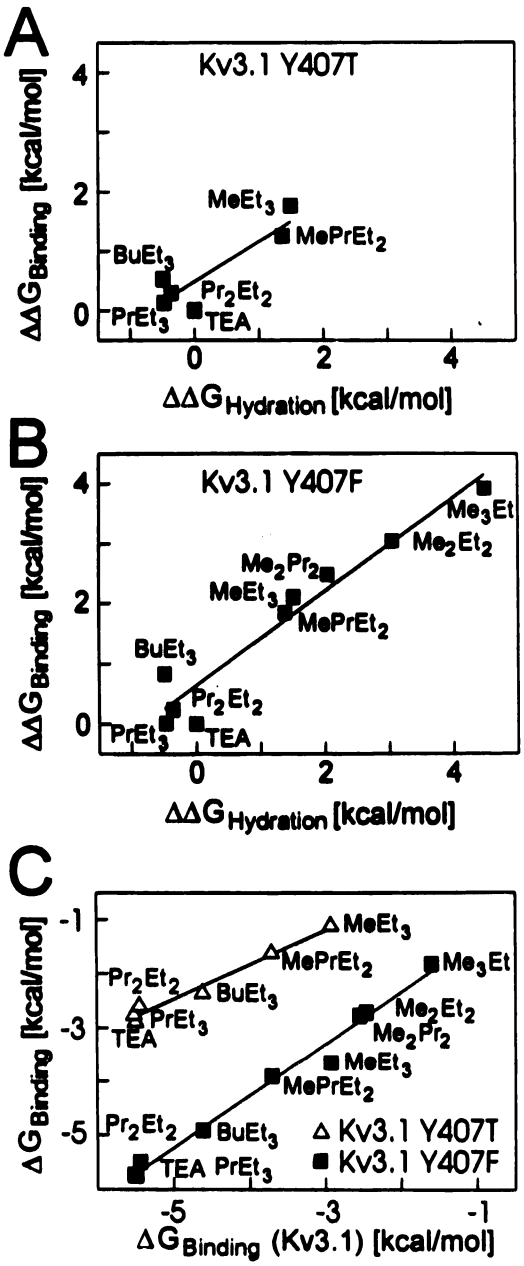

Fig. 6. Correlation of $\Delta\Delta G_{Binding}$ with $\Delta\Delta G_{Hydration}$ for Kv3.1 Y407T (A) and Kv3.1 Y407F (B) and $\Delta G_{Binding}$ of QA ions to Kv3.1 wild-type with $\Delta G_{Binding}$ to Kv3.1 mutants. *Solid lines*, regression lines for all QA ions tested. See legend of Fig. 4 for details. C, The leftward shift of the regression line for the Kv3.1 Y407T mutant from the diagonal indicates a reduced affinity in comparison to Kv3.1 wild-type, whereas a slight rightward shift of the regression line for the Kv3.1 Y407F mutant indicates an increased binding affinity. A higher $\Delta G_{Binding}$ value indicates a reduced binding affinity.

The mechanism of TEA selectivity. The selectivity for TEA over other QA ions at the external TEA receptor cannot be explained in terms of volume, surface area, diameter, or charge density of the QA ions (2, 4, present study). A cation π-electron interaction most probably dominates the binding energy between the QA ion (cation) and the K^+ channel (the aromatic residue). The mechanism for cation π-electron interactions has been extensively investigated for a cyclophane-based host and a variety of organic nitrogen compounds (18). However, these data cannot explain the selectivity of K^+ channels for TEA. We recently found that $\Delta\Delta G_{Binding}$ of QA ions correlates with $\Delta\Delta G_{Hydration}$ (4). In the present study, we demonstrate that a similar correlation can be shown in a different channel (Shaker B T449Y) and in mutants of Kv3.1. We propose a general mechanism in which water that is bound to the QA ions (19) must be partially removed to establish the cation π-electron interaction. The energy required for dehydration is different for different QA ions, whereas the subsequent energy provided by the binding site may be similar. Consistent with the idea that QA ions must be partially dehydrated to bind, gas phase calculations of interaction energy between a QA ion (TMA) and phenol/benzene (analogues of Tyr/Phe) were consistent with our experimental findings (9). Similarly, only when solvent effects are included does the calculated selectivity sequence for Li^+, Na^+, K^+, and Rb^+ parallel that seen in voltage-gated K^+ channels (20).

The conclusions depend on the computed $\Delta G_{hydration}$ values (Table 1); we therefore attempted to assess their reliability. A comparison of the calculated and experimental values of $\Delta G_{hydration}$ of ions is difficult. Absolute values of $\Delta G_{hydration}$ cannot be directly measured for individual ions, only for salts or relative to another ion, and the free energy for the ion of interest must be extracted from the measured quantities with the help of some assumptions (21, 22). To our knowledge, $\Delta G_{hydration}$ values for the QA ions used in the present study are not available in the literature. However, hydration enthalpies (ΔH) have been reported for TMA (−59.99 kcal/mol) and TEA (−57.12 kcal/mol) (23). It is also known that the total hydration entropy contribution of ions constitutes ~10% of their hydration enthalpy (21). The above assumptions allows estimates to be made of free energies of solvation for TMA and TEA (−54 and −51.3 kcal/mol, respectively), which are in reasonable agreement with the computed values (Table 1).

The lack of correlation of $\Delta G_{Hydration}$ with volume or surface area of the QA ions (Table 1) also needs to be commented on. In the case of charged solutes such as QA ions, electrostatic interaction of the solute with the surrounding water molecules makes by far the dominant contribution to $\Delta G_{Hydration}$. The exact three-dimensional shape of the molecule (chemical structure and conformation) plays a major role in determining that interaction term and outweighs the volume and surface area effects. For example, Me_2Et_2A and Et_3A (see Table 1) have comparable volumes and surface areas, but the latter hydrates more strongly by 2.78 kcal/mol, probably because its structure (a proton in the place of a methyl group) allows closer approach of the water molecules to the charge center (the nitrogen atom) and therefore tighter interaction.

Our data are in good agreement with results obtained with mutations in Shaker B and Kv1.1, showing that aromatic residues are important for high affinity TEA binding (1, 17). In agreement with these reports, the rank order of TEA sensitivity of Kv3.1 is Phe > Tyr. There are two possible explanations for the difference between TEA binding to the Phe and Tyr mutants. First, the cation π-electron interaction may be stronger for Phe than for Tyr. To our knowledge, no published quantum chemical calculations for the interaction energy of TEA with a ring of four Tyr or Phe exist to support this hypothesis. Second, the hydroxyl group of the Tyr may create a hydrophilic environment that hampers dehydration of the QA ion. Most important, the rank order of potency of

QA ions was similar for Phe, Tyr, and Thr substitutions, consistent with the interpretation that QA selectivity is determined by the dehydration energy of QA ions.

Given the importance of a cation π-electron interaction for the K^+ selectivity of K^+ channels (20), changes of Rb^+ versus K^+ conductance were to be expected for the different mutations in Kv3.1. The close agreement between the conductance and K_D changes observed in Kv3.1 (4, present study) and Shaker B (1, 12, present study) emphasize the critical role of this position in QA ion block and as a selectivity filter for permeating ions.

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Send reprint requests to: Dr. W. Jarolimek, I. Physiologisches Institut, Universität Heidelberg, Im Neuenheimer Feld 326, D-69120 Heidelberg, Germany.