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A residue W756 in the P-loop segment of the sodium channel is critical for primaquine binding

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ABSTRACT

Our study on the wild-type and mutants of the voltage-dependent sodium channel in the rat skeletal muscle Na_v 1.4 was to examine the possible binding site of primaquine PQ by using an experimental approach. We used a standard voltage-clamp in oocytes. Previously, we had demonstrated that PQ blocks the voltage-dependent sodium current in rat myocytes and that this blocking is concentration-dependent and voltage-independent. The direct-site mutagenesis in the P-loop segment W402C, W756C, W1239C, W1531A at the outer tryptophan-rich lip, and D400C, E758C, K1237C, A1529C of the DEKA locus helped us to identify residues playing a key role in aminoquinoline binding. In full agreement with our computed results, where a 1000-fold reduction of inhibition was measured, the tryptophan 756 is crucial for the reversible modulating effects of PQ. The W756C decreased the blocking effect of PQ in voltage-clamp assays. This new binding site may be important to the development of new drugs that modulate sodium inward currents.

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1. Introduction

Sodium channels are important for several processes in tissues, such as the transmission of the action potential, communication, secretion, or excitation–contraction coupling in muscle cells. They constitute molecular arrangements inside the cell membrane and facilitate the swift passage of millions of ions per ms per channel. They cover four different spans called domains DI to DIV, and each has six transmembrane segments S1 to S6. Between segments S-5 and S-6 of all 4 domains lies the P segment pore loop of the α subunit that forms the actual pore of the channel (Hille, 2001). In this central cylindrical cavity lies the selectivity filter. It has been proposed that at least two rings of amino acid residues delineate this filter motifs that are called the DEKA; aspartic acid, glutamic acid, lysine, and alanine and the EEDD; glutamic acid and aspartic acid. Although the crystal structure of the pore has not yet been elucidated, it is known that the spatial arrangements and the sequence composition of the locus vary according to the specific function of each channel type to filter incoming cations that differ in their ionic charges, radii, and solvation energies (Tikhonov and Zhorov, 2005; Yamagishi et al., 2001).

Primaquine PQ, an 8-aminoquinoline, has been used for several years in the treatment of relapsing malaria produced by *Plasmodium vivax and P. ovale*. Clinically, the PQ shows relevant adverse effects, commonly associated with alterations of conduction, depression of the cardiovascular function myocardial contractility, and rhythm disturbances. It has been suggested that these effects are associated with the blocking of sodium channels (Orta-Salazar et al., 2002) by reversibly binding to the open states. The reverse process of unbinding occurs either when channels move between inactivated states or from an inactivated state to a closed state. The PQ blocks several isoforms Nav 1.2, 1.4, 1.5, and 1.7 with different potencies. Though substantial literature has been developed about the biophysical and pharmacological characteristics of the sodium channel, the effects of several relevant Na^+-channel modulators, in particular the aminoquinolines, remain unclear (Orta-Salazar et al., 2002).

The most common binding sites in the pore of sodium channel are i) the tetrodotoxin TTX binding site outside the channel mouth Y401 and ii) the local anesthetic binding site LABS below the DEKA locus at the intracellular mouth F1579. These are the sites that lie within the pore region and are occupied by specific blockers, which decrease the amplitude of the sodium current (Hanck et al., 2009; Sunami et al., 2000). We previously have hypothesized that aminoquinolines may use a different binding site inside the sodium channel, besides the hitherto known sites binding certain toxins, i.e. TTX, brachotoxin, μ-conotoxins, and ATX, and local anesthetics, such as lidocaine.

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The aim of our study was to examine the possible binding site of PQ using direct-site mutagenesis in transfected oocytes from *Xenopus laevis*. We observed that the PQ binds preferentially in the external mouth of the channel, specifically on the W756 of Domain II. This binding to this site was strongly reduced when the W756 was mutated to a cysteine residue.

2. Materials and methods

Adult *X. laevis* female frogs Xenopus I, Ann Arbor, MI, USA were anesthetized by immersion in 0.2% MS-222. The stage V and VI oocytes were surgically removed, placed in OR-2 buffer containing (in mM) 82.5 NaCl, 2.5 KCl, 1 $MgCl_2$, and 5 HEPES 4-2-hydroxyethylpiperazine-1-ethanesulfonic acid, pH 7.6, and then treated with collagenase 1.3 mg/ml to remove the follicular membrane. The nuclei of the oocytes were injected by using a nanoliter automatic injector model A203XVY (WPI, Sarasota, FL,USA) with 15 to 25 ng of rNa_v Y401C, F1579A, D400C, E758C, K1237C, A1529C, W402C, W756C, W1239C, W1531A, and the $rNa_v1.4$ α-subunit cDNA clones (Li et al., 1999b; Nuss et al., 1995). Eggs were then maintained at 18 °C in ND-96 solution in mM, 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES, and 1 $CaCl_2$, pH 7.6 supplemented with 0.5 mM theophylline, 0.5 mM pyruvate, and 50 μg/ml gentamicin for up to 3 days before recording. Dr. Eduardo Marban kindly provided the α-subunit $rNa_v1.4$ cDNA vector GW1H (British Biolabs, Oxford, UK). Some of the mutants were kindly provided by Dr. Robert Tsushima (University of York).

2.1. Electrophysiological recording in oocytes

Oocytes were placed in a 1 ml recording chamber and continuously superfused with a barium-containing solution at a flow rate of approximately 500 μl/min. Two electrode voltage-clamp recordings were made at room temperature (20 to 22 °C) using an OC-725 C amplifier (Warner, New Haven, CT). The electrodes were pulled on a horizontal puller P-97 (Sutter Instruments, Novato, CA). The agarose-cushion electrodes, filled with 3 M KCl (Schreibmayer et al., 1994), were used to achieve a final resistance of 0.6 to 1.2 MΩ. The sodium-current signals were digitized at a sampling rate of 10 kHz by an analog-to-digital converter Digidata 1200 (Axon Instruments, Foster City, CA) and stored on a computer for analysis with pClamp software Version 8.02 (Axon Instruments, Foster City, CA). The sodium currents INa^+ were generated by step depolarizations from a holding potential of −100 mV at 0.1 Hz, unless otherwise indicated. The amplitude of the expressed INa^+ was typically 1 to 10 μA. Only oocytes with an INa^+ peak lower than 7 μA were used in the study to minimize voltage-clamp errors (Li et al., 1999a). The current–voltage I–V relationships were determined from peak currents generated by 30-ms, 10-mV steps from a holding potential of −100 mV to +50 mV. The voltage dependence of the steady-state inactivation of the Na^+ channels was determined by a two-pulse protocol. A first variable-voltage conditioning pulse that lasted 1000 ms was used to inactivate different fractions of the sodium channels. Two milliseconds later, a second 25 ms long test pulse was used at −20 mV. Data were normalized to the maximum sodium current recorded during the test pulse. The recovery from inactivation was examined by using a 500 ms conditioning pulse at −20 mV from a holding potential of −100 mV to inactivate sodium channels, followed by a recovery interval of variable duration Δt = 1 to 1000 ms and a test pulse at −20 mV. The use-dependent blocking of Na^+ channels was evaluated by a train of twenty 20 ms long pulses at −20 mV at 1, 2, or 5 Hz from a holding potential of −100 mV. The current amplitudes for the INa^+ in each pulse in the train were normalized to the INa^+-peak from the first pulse in each train. The average data for recovery from inactivation were later best fit by a double or triple exponential as needed of the formula: $y = A1^* \exp -t/\tau1 + A2^* \exp -t/\tau2 + A3^* \exp -t/\tau3 + y0$, where *A1*, *A2 and A3* are amplitude terms, *t* is time, τ1, τ2 and τ3 are time constants for the fast and slow and ultraslow inactivation phases, and y0 is the amplitude of the steady-state component. Activation and inactivation plots were generated by dividing the INa + peak total amplitude at the end of the voltage pulse, measured at a given potential, by the difference between the measured and reversal potential. The average from the activation data were best fitted with a Boltzmann distribution equation $G/G_{max} = 1/\{1 + \exp[V - V_{½}/k] + A\}$, where G is the conductance and G_{max} the maximum conductance, A the baseline, $V_{½}$ is the potential at which half the channels are activated, and k is the slope factor. For the inactivation plots $y = A_1/\{1 + \exp[V_m - V_{m½}]/k\} + A_2$, where y is the normalized INa^+, A_1, the baseline, A_2, amplitude of the component when not inactivated, V_m, the membrane potential; $V_{m½}$, the voltage of half-maximum inactivation, and k a slope factor.

2.2. Statistics and data analysis

The results are the mean ± S.E.M. The differences between mean data were analyzed with a paired or unpaired Student's *t*-test as appropriate. All the currents were analyzed using the pClamp version 10.2 software (Axon Instruments, Foster City, CA). The graphs were built using Sigmaplot v. 11(SPSS,Inc., Chicago, Il). The dose–response curve for the PQ effects was adjusted by the Origin 8.02 program (OriginLab Corp., Northampton, MA, USA) to calculate its EC_{50} value and 95% confidence limits CL according to the model $INa^+ = INa^+ max/1 + \{EC_{50}/[primaquine]^n\}$. A $P<0.05$ was used to denote statistical difference between groups.

3. Results

Fig. 1 shows the potency of several concentrations of PQ on the different sodium-channel isoforms Nav 1.2; 1.4; 1.5 and 1.7. PQ has a modulating effect on the sodium currents in those isoforms. We also show the chemical structure of primaquine with the molecular electrostatic potential.

To explore the possible union site for PQ, we did produce several site-directed mutagenesis in the P-loop of the four domains of the Nav 1.4 channels the amino acid identity between isoforms was about 81% in the region that we mutated. In Fig. 2 are the current–voltage relationships for the Y401C and F1579A. Those mutations were used to discard the possible union site to a well-known TTX binding site Y401 and that of the LABS F1579. At the bottom of Fig. 2 is shown the raw current taken at −20 mV, just before and after the perfusion of the drug. Because all mutant channels under PQ exposure show the same effect as seen on the WT, it is evident that the drug did not bind to our mutation sites. In both cases the drug has no effect on the voltage-dependence of activation or the reversal potential. Fig. 2C shows a diagram indicating the sites for several site-directed mutagenesis in the pore region of the channel. Those mutations were made to corroborate that the PQ has the same blocking behavior as in the WT. Fig. 2D shows a comparative chart showing that the PQ has the same modulating effect in all the mutations made. The peak current recorded was decreased on average 46%, except for the W756C, 16%, the K1237C, 28%, and the W1531A, 36%.

We previously demonstrated that the W756 is an important residue for binding toluene in Na 1.4 channels through hydrophobic attraction (Scior et al., 2009). To find the possible site or sites for PQ binding, we focused on the tryptophan-rich lip the area at the outer vestibule and the DEKA motif (Tsushima et al., 1997). Firstly, we screened for changes in the PQ blocking of W402C, W756C, W1239C, W1531A, and then the DEKA loci, D400C, E758C, K1237C, and A1529C. Those substitutions were less sensitive to the PQ blocking effect, indicating that the outer lip of the channel may play a role in the binding site of the drug. However, the W756C sodium-channel mutation shows only little blocking effect at the same drug concentration.

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