Induction of a fast inactivation gating on delayed rectifier Shab K⁺ channels by the anti-inflammatory drug celecoxib

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Key words: potassium channels, Shab, inactivation, celecoxib

Celecoxib is a drug designed to selectively inhibit COX-2, an inflammation-inducible cyclooxygenase isoform, over the constitutively expressed COX-1 isoform. In addition to this selective inhibition it is now known that celecoxib exerts a variety of effects on several types of ion channels, thus producing secondary physiological effects. In this work we demonstrate that at therapeutically relevant concentrations celecoxib interacts with Shab K⁺ channels specifically promoting a fast inactivation gating (without blocking the pore or significantly affecting other gating processes). At least two celecoxib molecules bind to each channel promoting a fast inactivation that develops from both open and closed states. Channel inactivation in turn causes a reduction of the size of I_K. Taken together, our observations show that in addition to its intended therapeutic target celecoxib is a useful tool to further study the mechanism of Shab channel inactivation.

Introduction

Celecoxib (hereafter referred to as Cx) is a non-steroidal anti-inflammatory drug designed to specifically inhibit COX-2, an inflammation-inducible cyclooxygenase isoform, and to thus minimize the harmful effects that inhibition of the constitutive COX-1 isoform exerts on the gastric mucose integrity. Despite its COX-2/COX-1 specificity, recent reports demonstrate that at therapeutic micromolar concentrations, Cx also interacts with several types of ion channels. The latter interactions are thought to mediate several unanticipated physiological effects of Cx such as reported anti-hypertensive and anti-tumor effects. Of particular note, it has been demonstrated that Cx interaction with ion channels may occur under conditions in which COX-2 expression is not induced.3-5

Regarding K⁺ channels, it has been reported that Cx increases the current through EAG Kᵥ7 channels⁵ while it inhibits both the transient and sustained I_K of rat amacrine and ganglion cells.⁴ Also, it has been demonstrated that Cx interacts with delayed rectifier Kᵥ2,1 channels in a complex manner, blocking the pore and affecting the gating of activation, deactivation and inactivation.⁶

A comprehensive study of Cx interaction with channels is a necessary step to determine both its pharmacological scope and the diversity of the mechanisms by which this therapeutic drug is capable to interact with ion channels. In particular, we are interested in finding pharmacological tools that can be used to study the gating mechanisms of Shab channels. Thus, considering the previous reports regarding Cx effects on K⁺ channels we decided to study the interaction of this drug with Shab channels.

Kᵥ2,1 is the mammalian homolog of Shab and it has been previously reported that Cx interacts in a complex manner.⁵,⁷ However, it is well known that Kᵥ2,1 and Shab channels are not functionally equivalent, and therefore there was no reason to expect that Cx might interact with Shab in an identical manner. Functional differences between Kᵥ2,1 and Shab channels are not only quantitative but also qualitative. For example, it is known that in the absence of K⁺ ions, Kᵥ2,1 channels lose their selectivity but remain capable to conduct ions, while in contrast under the same conditions, Shab channels irreversibly cease to conduct ions.⁸,⁹ Further, regarding channel inactivation it is known that Kᵥ2,1 possesses a U-type slow inactivation¹⁰ while Shab does not.¹¹

In the current study Cx interaction with delayed rectifier Drosophila Shab channels was studied following transient expression in insect SF9 cells, thus avoiding the complexity of interpretation inherent to studies with native cells, which typically present several distinct K⁺ conductances. We found that Cx specifically accelerates the otherwise slow inactivation gating of Shab. While the unmodified Shab presents a slow inactivation that develops over a time scale of seconds,¹² the Cx-modified channel presents a fast inactivation that develops in a time scale of milliseconds,
and as a consequence, \( I_K \) amplitude is significantly reduced. Our observations thus show for the first time that, in addition to its varied physiological effects, Cx is a valuable tool to study the inactivation gating of Shab channels.

**Results**

While some K⁺ channels such as Shaker present both fast and slow inactivation mechanisms, wild-type Shab channels only present a slow inactivation process that becomes significant only when the membrane is depolarized for several seconds.12 However, after the addition of celecoxib to the extracellular solution, Shab channels inactivate fast, within a millisecond time scale. This is shown in Figure 1A, which presents \( I_K \) (see Materials and Methods) evoked by either a 50 (left part) or a 300-ms pulse to 0 mV (right part) in the presence and absence of 10 μM Cx. It is important to note that in contrast to control channels which do not present a significant inactivation during the applied pulses, in the presence of Cx \( I_K \) rises to a peak with a kinetics equal to that of control \( I_K \), and thereafter decays monotonically as channels inactivate (see later), so that a ~63% inactivation was achieved by the end of the 50-ms pulse and was essentially complete (~90%) by the end of the 300-ms pulse. The inset in the right part shows that the Cx-promoted inactivation follows a single exponential time course (solid line through the sample points of the decaying fraction of the Cx-inactivated channels (50–60%), recovered during a second, much slower, phase with a time constant of 256 ms yields about a 40–50% recovery. The remainder of either 142 or 86 ms, at either -100 or -120 mV, respectively. On the other hand, at -80 mV recovery is slower, and presents two exponential phases. The first phase (Fig. 3B) with a time constant of 256 ms yields about a 40–50% recovery. The remaining fraction of the Cx-inactivated channels (50–60%), recovered during a second, much slower, phase with a time constant of 2.6 sec (Fig. 3C; see Discussion). Finally, note that because of the time course of recovery, if activating pulses are delivered from the HP of -80 mV at frequencies ≥ 4 Hz, then there will be a use-dependent reduction of \( I_K \) (not shown).

In addition to promoting the observed strikingly fast inactivation, Cx addition also reduces \( I_K \) amplitude (Fig. 1A). Since it is well known that inactivation per se reduces the size of currents the question arises as to whether the observed \( I_K \) reduction could be entirely accounted for by the Cx-promoted inactivation. Alternatively, a fraction of the observed \( I_K \) reduction could be accounted for by a possible pore blockage.

In order to address these issues the experiments shown in Figures 4 and 5 were performed. First, \( I_K \) reduction was assessed by applying an I–V pulse protocol. Figure 4A left part presents control \( I_K \) evoked by 50 ms pulses from -50 to +50 mV applied in 10 mV steps every 17 sec, to allow a full recovery from
inactivation between pulses at -80 mV. Thereafter, the cell was extensively perfused with a solution containing 50 μM Cx and the I–V protocol was applied (middle part). I_x was found to be reduced at all voltages. Subsequently, the cell was perfused for 1-min with the control solution and the traces in right part were recorded. There was a complete recovery, which demonstrates the reversibility of the Cx effect. Note that the fast reversibility of the Cx effect agrees with a direct interaction of the drug with the
Figure 4C shows the fractional reduction of \( I_k \) as a function of \([Cx]\) at 0 mV, determined from at least four independent experiments as in Figure 4A at each \([Cx]\). The line is the fit of the points with a Hill equation with \( n_{Hill} = 1.5 \) and apparent \( K_d \) of 10.6 \( \mu M \). Although slightly smaller, the obtained \( n_{Hill} \) agrees with the value previously obtained from the \( k \) vs. \([Cx]\) curve. Taken together these values suggest that the Cx:channel stoichiometry is at least 2:1.

Channels (see Discussion). The latter is best observed in the I–V relationship in Figure 4B. The inset shows the fractional reduction of \( I_k \) as a function of \( V_m \). \( I_k \) reduction reaches an essentially constant value at voltages where activation is complete (indicated by the dashed line). This, along with the hydrophobicity of Cx, suggests that the voltage dependence of \( I_k \) reduction arises from channel inactivation.

Figure 2. Celecoxib does not affect the rate of channel deactivation. (A) Left part, superimposed \( I_k \) recorded either in control conditions (50 mM K\(^+\)) or in the presence of Cx (smaller trace 15 \( \mu M \) Cx + 15 mM K\(^+\)). \( I_k \) was evoked by a 0 mV pulse followed by a repolarization to -100 mV. Right part, superimposed tail \( I_k \) at -100 mV of the traces in the right part. \( I_k \) were scaled to compare their time course. (B) Deactivation time constant obtained by fitting a single exponential to the tails obtained from at least four independent experiments as in (A and B), control: closed circles, Cx: open circles.

Figure 3. Recovery from Cx-promoted inactivation. (A) Extent of recovery after a 200 ms repolarization to -120 mV. (B) Extent of recovery (Er) as a function of the time between pulses, at the indicated repolarization potentials. Er was determined as: \( Er = (I_{peak,2} - I_{end,2})/(I_{peak,1} - I_{end,1}) \), where \( I_{peak} \) is the peak \( I_k \) at either the first or the second pulse, and \( I_{end} \) is \( I_k \) at the respective pulse end, \([Cx]\) = 25 \( \mu M \). The lines are the fit of the points with the equation: \( fr = fr_{max}(1 - \exp(-t/\tau)) \), with parameters, -80 mV: \( \tau = 256 \) ms, \( fr_{max} = 0.54 \); -100 mV: \( \tau = 143 \) ms, \( fr_{max} = 0.9 \); -120 mV: \( \tau = 86 \) ms, \( fr_{max} = 0.98 \). (C) Slow phase of recovery at -80 mV. The line is the fit of the points with the function: \( Er = A*(1 - \exp(-t/\tau)) + C \) with \( A = 0.45 \), \( C = 0.55 \), \( \tau = 2.6 \) sec. The points are the mean of that least four experiments at each \( V_m \).
The above observations suggest that $I_k$ reduction is entirely due to the Cx-promoted inactivation. Moreover, considering its size, hydrophobicity and the lack of effect observed when applied intracellularly, it can be hypothesized that if Cx directly blocks K⁺ channels it is likely via binding to the external vestibule of the pore, above the narrow and hydrophilic selectivity filter (see Discussion). If this were the case, Cx should compete with the binding of external TEA, since it is known that TEA binds to the outer vestibule of the pore at the entry of the selectivity filter.16,17

The aim of the experiments in Figure 5 was to determine if Cx competes with the binding of external TEA.

Figure 5A illustrates TEA block of control, unmodified, channels at 0 mV. Note that 15 mM TEA blocks ~50% of control $I_k$. As shown in Figure 5B, to assess TEA blockage of Cx-modified channels cells were first perfused with a solution containing 50 μM Cx and channels were activated with a 0 mV/50 ms pulse (trace labeled 50 μM Cx). Thereafter, cells were perfused with a solution containing 50 μM Cx plus 15 mM TEA and channels activated as previously (bottom trace, as indicated). The traces clearly show that TEA blocks both control and Cx-modified channels with the same potency, indicating that TEA blockage is not affected by Cx binding to the channels. The latter is demonstrated by the dose-response curve in Figure 5C, which was obtained from four experiments as in Figure 5A. The line is the fit of the points with a Hill equation with $n_Hill = 1.5$ and $K_d = 10.6 \mu M$ (see text).

Discussion

After celecoxib was added to the external solution Shab channels were found to inactivate fast. Inactivation presents a rate constant $k$ whose variation with [Cx] indicates the binding of at least two Cx molecules per channel. Interestingly, membrane
In the case of local anesthetics for the inhibition of Na\(^+\) channels it has been proposed that there are both hydrophilic and hydrophobic pathways within the pore,\(^1\) thus a possibility is that Cx could block the Shab pore through a hydrophobic pathway, thus explaining the lack of competition between Cx and TEA binding. However, as far as we know, such hydrophobic pathways have not been documented in K\(^+\) channels. Therefore, based on the observations here presented, and knowing that inactivation brings a concomitant reduction of current amplitude, as exemplified by the large increase of both Shaker and Na\(^+\) channel currents seen when inactivation is removed,\(^1\)\(^,\)\(^2\)\(^,\)\(^3\)\(^,\)\(^4\) we conclude that I\(_K\) reduction is entirely due to the Cx-promoted inactivation.

The above conclusion is reinforced by the I\(_K\) simulations presented in Figure 6. In the absence of comprehensive studies of the Shab activation pathway, we relied on the kinetic studies of Shaker channels,\(^5\) and thus simulated control I\(_K\) at 0 mV using a linear scheme containing five closed states C an a single open state O, as depicted below:

\[
O \rightarrow I_1 \rightarrow I_2
\]

It is pertinent to point out that, as in the case of the ball-and-chain inactivation, the presence of I\(_K\) does not affect the time course of I\(_K\) during a step depolarization, as the transition I\(_K\) to I\(_K\) is slow, and only affects the recovery rate. Therefore, for simplicity, in the following discussion (Fig. 6) we will not consider state(s) I\(_K\).

Our observations strongly indicate that Cx does not block the Shab pore. Thus, I\(_K\) reduction was not affected by the external [K\(^+\)], the voltage-dependence of I\(_K\) reduction indicated that it was coupled to the underlying inactivation of the channels, and importantly Cx binding did not affect TEA blockage.

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\[
C_5 \rightarrow C_4 \rightarrow C_3 \rightarrow C_2 \rightarrow C_1 \rightarrow O \quad \cdots \quad (1)
\]

The scheme does not include deactivation rates, because at 0 mV they can be neglected. Trying to fit the numerical solution of Scheme 1 to the normalized recorded I\(_K\) we found that: (a) it is not possible to fit Shab I\(_K\) (at any voltage) using Hodgking and Huxley (HH) schemes (with either five or four closed states), where all the rate constants monotonically decrease in the order 5:4:3:2:1 (not shown); instead (b) in order to reproduce I\(_K\), the rate of the first transition, \(\alpha_1\), must be the slowest one of the

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**Figure 5.** External TEA block in the presence of celecoxib. (A) Control I\(_K\) evoked by a 0 mV/50 ms pulse superposed to I\(_K\) recorded with 15 mM TEA in the external solution, as indicated. TEA blocked ~50% of control I\(_K\). (B) I\(_K\) evoked by a 0 mV/50 ms pulse applied in the presence of 50 \(\mu\)M Cx, superimposed to I\(_K\) recorded with 50 \(\mu\)M Cx plus 15 mM TEA in the external solution, as indicated. TEA blocked ~50% of the Cx-modified I\(_K\). (C) I\(_K\) block as a function of [TEA]. Closed circles: TEA block of control I\(_K\), obtained from experiments as in (A). Triangles: TEA block in the presence of 50 \(\mu\)M Cx, obtained as in (B). The line is the fit of the control points (closed circles) with a Michaelis-Menten equation with K\(_d\) of 11 mM. The points are the mean ± SEM of at least four experiments at each [TEA].
Figure 6. I_K simulations and G-V curve. (A) Normalized control I_K at 0 mV. The line is the fit of the sample points of the recorded I_K with the numerical solution of kinetic model 1 (see text). (B) Control I_K in (A) superposed to I_K recorded in the presence of 10 μM Cx, as indicated. The dashed line is I_K simulated with kinetic Scheme 2. Clearly, inactivation from only the open state does not reproduce I_K in the presence of Cx. (C) Control I_K superposed to I_K recorded in the presence of 10 μM Cx in (B). The lines are the fit of the sample points with the numerical solution of either Scheme 1 (control) or Scheme 3 (I_K recorded with 10 uM Cx present). Inactivation from both the open and closed states reproduces Cx effect on I_K. (D) Relative chord conductance (G/G_{max}) in either control conditions or in the presence of 10 μM Cx, as indicated. Chord conductance was calculated as: G(V_m) = I_K(V_m)/(V_m - V_K), where I_K is the current measured at either the end of a 40 ms pulse of the indicated V_m amplitude (control), or the maximal peak I_K measured in the presence of 10 μM Cx. V_K is the calculated Nernst potential of K⁺ (5 mM K⁺, V_K = -79 mV). The lines are the fit of the points with a Boltzmann equation: G/G_{max} = 1/[1 + \exp(-zF(V_m - V_{1/2})/RT)], with parameters: control: V_{1/2} = -20 mV, z = 2.1; and 10 μM Cx: V_{1/2} = -2 mV, z = 1.6. F, R, T have their usual meaning. The points are the mean ± SEM of 4 independent experiments. (E) Simulation of the time course of inactivation (kinetic Scheme 3, 0 mV). I_c is the inactivation from closed states (C_3 - C_1), I_o is the inactivation from state O; I_T = I_c + I_o. Traces labeled Control and Control I_K + Cx are the simulated I_K in either control conditions or with 10 μM Cx, as in (C).
pathway. This rate, $\alpha_f$, was chosen to fit the condition to Schopka and Sigsworth 1998: 

$$\alpha_f = 1/\tau_{act}$$

where $\tau_{act}$ is the time constant obtained by fitting an exponential of the form $I_{K,act} = I_{max}(1 - \exp(-t/\tau_{act}))$ (not shown) to the upper half of the current ($I_{K,act}$). The remaining rates ($\alpha_i - \alpha_5$) were adjustable parameters obtained under the condition: $2\alpha_i^{-1} \approx 8$, for $i \neq 5$. Finally, for simplicity, these rates ($\alpha_i - \alpha_5$) were constrained to decrease as in HH kinetics, following the ratio 4:3:2:1.

After fitting the numerical solution of Scheme 1 to normalized control $I_K$ (solid line through the sample points in Fig. 6A, see figure legend), the effect of adding an inactivated I state on $I_K$ amplitude was analyzed. First, we considered the case in which inactivation proceeded only from the open state, with a rate $k$ equal to that obtained from the decay phase of $I_K$ in the presence of 10 $\mu$M Cx, as in Figure 1A ($k = 0.02$ (ms$^{-1}$)), keeping rates $\alpha_i$ to $\alpha_5$ as in Figure 6A, according to the Scheme 2:

$$C_5 \rightarrow C_4 \rightarrow C_3 \rightarrow C_2 \rightarrow C_1 \rightarrow O \rightarrow I \quad \ldots \quad (2)$$

Figure 6B presents control $I_K$ superimposed to $I_K$ in the presence of 10 $\mu$M Cx. The solid line is the fit of control $I_K$ with the numerical solution of Scheme 1, as in A. The dashed line is the simulation of Scheme 2. As expected, inactivation from the open state significantly decreases $I_K$; however the decrease is smaller than that experimentally observed. The latter indicates that Cx promotes inactivation both from open and closed states, in agreement with the left shift of the $h$ curve.

Considering that inactivation is coupled to channel activation, we considered kinetic schemes where the Cx-promoted inactivation proceeded from states located at the right of the first transitions in the activation pathway, with rate equal to that measured from the open state ($k = 0.02$), multiplied by an adjustable factor $b$ ($k b^* b$). $b$ can be viewed as an allosteric factor of inactivation from closed states.$^9$ After testing several possibilities we ended up with the following model of Cx-Shab interaction:

$$C_5 \rightarrow C_4 \rightarrow C_3 \rightarrow C_2 \rightarrow C_1 \rightarrow O \rightarrow I \quad \ldots \quad (3)$$

Figure 6C presents $I_K$ in B fitted with either the numerical solution of Scheme 1 (control $I_K$) or Scheme 3 ($I_K$ in the presence of Cx, $b = 12$), as indicated. Altogether, these results show that $I_K$ reduction can be explained as entirely arising from channel inactivation. The effect of the Cx-promoted inactivation on the Shab $K^+$ conductance is further discussed below.

It is known that removal of inactivation, for example that produced by the action of proteolytic enzymes on Na$^+$ channels, shifts the G-V curve to the left along the V-axis.$^{3,23}$ Moreover, Gonoi and Hille 1987,23 have observed that this shift is predicted by most inactivation models. Therefore, it is expected that the opposite process, namely the induction of a fast inactivation gating, as that exerted by Cx on Shab channels, should shift the G-V curve to the right.

The data in Figure 6D demonstrates that in agreement with the above prediction, Cx shifts the Shab G-V curve towards depolarized potentials, without affecting the slope of the peak conductance curve (see figure legend). This voltage shift is expected since inactivation decreases the amount of open channels.$^{13,23}$

The above is illustrated in Figure 6E, which compares the time course of simulated $I_K$ (as indicated, Fig. 6C) with the time course of the simulated Cx-promoted inactivation. $I_{act}$ is the fraction of the total inactivation ($I_{act} = I_{open} + I_{closed}$) that develops from the open state (kinetic Scheme 3), while $I_{inact}$ is the fraction that develops from closed states (C$_3$ to C$_0$), as indicated. Note that at early times, before peak $I_K$ in the presence of Cx is reached, $I_K$ is dominated by $I_{act}$, while at later times inactivation is dominated by $I_{inact}$. Altogether, this produces a steady increase of the total amount of inactivation during the pulse and therefore reduces the peak $K^+$ conductance.

In other words, at 0 mV when peak $I_K$ is reached in the presence of Cx, $\approx 20\%$ channels have already become inactivated from closed states and $\approx 15\%$ from the open state, yielding $\approx 35\%$ inactivation, which translates into a $\approx 50\%$ peak $I_K$ reduction, compared with control $I_K$ at pulse end in the absence of Cx (the remaining difference ($\approx 15\%$) comes from non-inactivated channels that are still traveling along the activation pathway).

Finally, it should be mentioned that both the easiness with which Cx effects are reverted and the lack of COX-2 in S9 cells$^{24}$ indicate that Cx promotes Shab inactivation by binding directly to the channel.

A comparison with other channels. In spite of presenting high COX-2/COX-1 specificity, Cx affects several ion channels. Thus, Cx reduces the increment of the TTX-resistant $I_{Na}$ that occurs after tissue injury in rat dorsal root ganglia (DRG) neurons. The inhibition seems to be a consequence of the effect that Cx exerts on PGE$_2$ and CGRP levels on these neurons.$^{25}$ Cx also exerts a direct inhibitory effect on both TTX-sensitive and resistant $I_{Na}$, contributing to decrease neural excitability. Additionally, Cx inhibits $I_{Na}$ in rat amacrine and ganglion cells, decreasing their spontaneous firing rate.$^4$ Cx also inhibits Ca$^{2+}$ channels. It has been reported that it depresses L-type $I_{Ca}$ of PC12 cells and that this capability could underline the anti-tumor effect of the drug.$^3$ A direct Cx inhibition of L-type $I_{Ca}$ has also been documented in vascular smooth muscle cells in which this inhibition contributes to the Cx vasodilatation effects.$^5$

As stated above, our observations show for the first time that Cx induces a fast inactivation gating that decreases $I_K$ amplitude without blocking Shab channels. Further studies are needed to determine the structural basis of the varied effects of celecoxib on different ion channels, as well as the range of physiological effects of this drug.

Materials and Methods

Cell culture and Shab channel expression. S9 cells grown at 27°C in Grace’s media (Gibco) were infected with a multiplicity of infection of $\approx 10$ with a baculovirus containing Shab (dShab 11) $K^+$-channel cDNA as reported.$^9,27$ Experiments were conducted 48 h post-infection.
Electrophysiological recordings. Currents were recorded under whole-cell patch-clamp with an Axopatch 1D amplifier (Axon Instruments). Except where indicated, currents were filtered on-line at 5 kHz, and sampled at 100 μsec/point, with a Digidata 1322A interface (Axon Instruments). Electrodes were made of borosilicate glass (KIMAX 51) pulled to a 1–1.5 MΩ resistance. About 80% of the series resistance was routinely compensated. The holding potential (HP) was -80 mV.

Solutions. The internal solution contained (in mM): 30 KCl, 90 KF, 2 MgCl₂, 10 EGTA-K, 10 Hepes-K. The external solution contained (in mM): 5 KCl, 140 NaCl, 10 CaCl₂, 10 Hepes-Na. The pH of all solutions was 7.2. Celecoxib, 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide, from Celebrex capsules was dissolved in dimethyl sulfoxide (DMSO). The DMSO added at the highest concentration of celecoxib tested (50 μM) did not have any effect on channel properties. Capsule excipients (www.medicines.org.uk) are inert on K⁺ channels ([Mg²+] was much less than 30 μM at the highest (Cx) employed).

Data analysis. Kinetic schemes where numerically solved and fit to the recorded Iᵢ with SCoP software (Simulation resources INC.). Results are expressed as mean ± SEM of the indicated number of experiments.

Acknowledgements

The authors thank DGAPA for postdoctoral scholarship to Imilla I. Arias-Olguin and Daniel Balleza. This work was supported by DGAPA grant IN221610.

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