UNDERLYING MECHANISM OF REGULATORY ACTIONS OF DICLOFENAC, A NONSTEROIDAL ANTI-INFLAMMATORY AGENT, ON NEURONAL POTASSIUM CHANNELS AND FIRING: AN EXPERIMENTAL AND THEORETICAL STUDY

C.W. HUANG¹, T.Y. HUNG², Y.K. LIAO³, M.C. HSU³, S.N. WU³,⁴

Diclofenac (DIC), a nonsteroidal anti-inflammatory drug, is known to exert anti-nociceptive and anti-convulsant actions; however, its effects on ion currents, in neurons remain debatable. We aimed to investigate (1) potential effects of diclofenac on membrane potential and potassium currents in differentiated NSC-34 neuronal cells and dorsal root ganglion (DRG) neurons with whole-cell patch-clamp technology, and (2) firing of action potentials (APs), using a simulation model from hippocampal CA1 pyramidal neurons based on diclofenac's effects on potassium currents. In the NSC-34 cells, diclofenac exerted an inhibitory effect on delayed-rectifier K⁺ current (I_{K(DR)}) with an IC₅₀ value of 73 µM. Diclofenac not merely inhibited the I_{K(DR)} amplitude in response to membrane depolarization, but also accelerated the process of current inactivation. The inhibition by diclofenac of I_{K(DR)} was not reversed by subsequent application of either naloxone. Importantly, diclofenac (300 µM) increased the amplitude of M-type K⁺ current (I_{K(M)}) while flupirtine (10 µM) or meclofenamic acid (10 µM) enhanced it effectively. Consistently, diclofenac (100 µM) increased the amplitude of I_{K(M)} and diminished the I_{K(DR)} amplitude, with a shortening of inactivation time constant in DRG neurons. Furthermore, by using the simulation modeling, we demonstrated the potential electrophysiological mechanisms underlying changes in AP firing caused by diclofenac. During the exposure to diclofenac, the actions on both I_{K(M)} and I_{K(DR)} could be potential mechanism through which it influences the excitability of fast-spiking neurons. Caution needs to be made in attributing the effects of diclofenac primarily to those produced by the activation of I_{K(M)}.

Key words: nonsteroidal anti-inflammatory drugs, diclofenac, delayed-rectifier K⁺ current, M-type K⁺ current, action potential, pyramidal neurons, simulations
for studying effects of potential neuroprotective compounds against different insults including excitotoxins, mitochondrial toxins, and oxidants (16-19). Previous observations in our laboratory have shown the ability of flupirtine, to suppress \( I_{k(DR)} \) in a concentration- and state-dependent manner in the NSC-34 cells (15). Dorsal root ganglion (DRG) neurons, conveying somatic and visceral sensory information from peripheral tissues to the spinal cord, exhibit voltage-gated K+ currents including \( I_{k(DR)} \) formed by Kv1-3 subunits, and \( I_{k(M)} \) generated by KCNQ2-5 subunits (20). Whether diclofenac had any effects on \( I_{k(DR)} \) or \( I_{k(M)} \) in these cells is unclear.

Therefore, the goal of this study was to evaluate the effects of diclofenac on ionic currents in differentiated NSC-34 motor neuron-like cells and DRG neurons. We found diclofenac not only diminished the \( I_{k(DR)} \) amplitude but also increased the time course of current inactivation. It stimulated the amplitude of \( I_{k(M)} \) in these cells. Our simulation modeling was allowed to predict that both blockade of \( I_{k(DR)} \) and stimulation of \( I_{k(M)} \) caused by diclofenac may synergistically act to affect the functional activity of mammalian neurons in cell culture or in vivo.

MATERIALS AND METHODS

Drugs and solutions

Diazepam, diclofenac, flupirtine, linopirdine, glibenclamide, and naloxone were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.), apamin, iberiotoxin and sea anemone toxin BDS-1 were obtained from Invitrogen (Carlsbad, CA, U.S.A.). All other chemicals were obtained from regular commercial chemicals and of reagent grade. Reagent water obtained from a Milli-Q ultrapure water purification system (Millipore, Bedford, MA, U.S.A.) was used in all experiments. The composition of normal Tyrode's solution is as follows (in mM): NaCl 136.5, KCl 5.4, CaCl\(_2\) 1.8, MgCl\(_2\) 0.53, glucose 5.5, and HEPES-NaOH buffer (pH 7.2).

Electrophysiological measurements

Cells were harvested with 1% trypsin/EDTA solution prior to the experiments and an aliquot of cell suspension was transferred to a recording chamber mounted on the stage of an inverted fluorescent microscope (CKX-41; Olympus, Tokyo, Japan). Cells were bathed at room temperature (20–25°C) in normal Tyrode's solution containing 1.8 mM CaCl\(_2\). Patch-clamp recordings in the whole-cell configuration were made with an RK-400 amplifier (Bio-Logic, Claix, France) (19, 21). Voltage-clamp protocols with either rectangular or ramp shapes were computer driven using an acquisition system (Digidata 1322A board; Molecular Device, Sunnyvale, CA, U.S.A.) and pClamp 9.2 (Molecular Devices). The latter device was equipped via PCMCIA interface (Molecular Devices). The data were stored online in a TravelMate-6253 computer (Acer, Taipei, Taiwan) at 10 kHz through Digidata 1322A interface (Molecular Devices). The latter device was equipped with Adaptec SlimSCSI card (Milpitas, CA, U.S.A.) via PCMCIA slot and controlled by pCLAMP 9.2 (Molecular Devices). Current signals were low pass-filtered at 3 kHz. Ion currents were generally analyzed by using Origin 8.0 (OriginLab, Northampton, MA, U.S.A.) or custom-made macros built in the Excel 2007 spreadsheets under Windows 7. The duration of action potential (AP) in response to brief current injection recorded under current-clamp mode was measured at 90% of repolarization.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

To detect the mRNA expression of Kv3.1 and M-type channel mRNA in differentiated NSC-34 cells, a semi-quantitative RT-PCR assay was performed. Total RNA samples were extracted from normal NSC-34 cells according to TRizol reagent protocol (Invitrogen). First-strand cDNA was synthesis using GoScriptTM Reverse Transcribease (Promega). The sequences of forward and reverse primers for \( kcn1 \) were as follows: \( 5' \)-TGACCTCCGTTCATCACCTGTTG-3'; and \( 5' \)-CTAGTGGGTTGGAGTGGTTT-3'. Forward and reverse primers for \( kcn2 \) were as follows: \( 5' \)-CCCTGAAAGTCCAAGAGCAG-3'; and \( 5' \)-AGGCCCATAGGTTGAGTGGTT-3'. Forward and reverse primers for \( kcn3 \) were as follows: \( 5' \)-GTGGCTTCAGCATCCACAAG-3'; and \( 5' \)-CTTTGCTGAGGAGTTCACTCATA-3'. Forward and reverse primers for \( kcn4 \) were as follows: \( 5' \)-CGATCACACTGACGCCATT-3'; and \( 5' \)-GAGGATTAGCTGTGCTGCCATCC-3'. Forward and reverse primers for \( kcn5 \) were as follows: \( 5' \)-ACAGTTTTTCAGGCAGGAGT-3'; and \( 5' \)-AGATGACCCTGACCTTTCCAG-3'. PCR cycling conditions were 35 cycles of 95°C for 2 min, 95°C for 20 s, and 72°C for 10 min. These PCR products were resolved on 2% agarose gels, analyzed on 1.5% (v/v) agarose gel containing ethidium bromide and then visualized under ultraviolet light. Optical densities of DNA bands were scanned and quantified by AlphaImager 2200 (ProteinSimple; Santa Clara, CA, U.S.A.).
The concentration-response data for diclofenac-induced inhibition of \( I_{\text{K(DR)}} \), measured from NSC-34 neuronal cells or dorsal root ganglion (DRG) neurons were adequately fitted using a modified form of the Hill equation. That is:

\[
y = \frac{1}{1 + \left(\frac{[C]}{K_{50}}\right)^n}\left(\frac{C_{\text{IC50}}}{C_{\text{IC50}} + [C]}\right)^m,
\]

where \( y \) is the relative amplitude of \( I_{\text{K(DR)}} \) measured at the end of depolarizing pulse; \([C]\) is the DIC concentration; \( IC_{50} \) and \( nH \) are the concentration required for a 50% inhibition and the Hill coefficient, respectively. Maximal inhibition (i.e., 1 – \( a \)) of \( I_{\text{K(DR)}} \) during cell exposure to DIC was estimated. The inactivation time constants of \( I_{\text{K(DR)}} \) with or without addition of DIC measured from the different levels of depolarizing voltages were determined by fitting the trajectory of each current trace with a single exponential. The nonlinear curve-fitting sets were performed using Origin 8.0 (OriginLab).

To evaluate the concentration-dependent effect of DIC on \( I_{\text{K(DR)}} \) in DRG neurons, the \( I_{\text{K(M)}} \) was elicited by membrane hyperpolarization to −40 mV from −10 mV. The data obtained at the end of hyperpolarizing pulses were fitted to another modified Hill equation:

\[
y = \frac{1}{1 + \left(\frac{[C]}{K_{50}}\right)^n}\left(\frac{C_{\text{IC50}}}{C_{\text{IC50}} + [C]}\right)^m,
\]

where \( y \) is the relative amplitude of \( I_{\text{K(M)}} \) measured at the end of hyperpolarizing pulse; \([C]\) is the DIC concentration; \( EC_{50} \) and \( nH \) are the concentration required for a 50% inhibition and the Hill coefficient, respectively. Maximal stimulation (i.e., 1 – \( a \)) of \( I_{\text{K(M)}} \) during cell exposure to DIC was estimated.

Values are provided as means ± standard error of the mean (S.E.M.) for \( n \) number of samples. The paired or unpaired Student’s \( t \)-test and one-way ANOVA with a least-significance difference method for multiple comparisons were used for the statistical evaluation of differences among means. A value of \( P<0.05 \) was considered to be statistically significant.

### Computational simulation

To mimic the effect of DIC on electrical behaviors of AP firing in central mammalian neurons, a theoretical model of hippocampal CA1 pyramidal neuron (22) was modified and implemented. In this model, rhythmic bursting was mathematically reconstructed from ionic processes that were formulated on the basis of the data experimentally obtained from CA1 pyramidal cells of brain slice preparation. The model mainly comprises a fast and transient \( Na^+ \) current, a persistent \( Na^+ \) current, a high-threshold \( Ca^{2+} \) current, two \( Ca^{2+} \)-activated \( K^+ \) currents, a transient outward \( K^+ \) current, a delayed rectifier \( K^+ \) current \( I_{\text{K(DR)}} \), and an M-type \( K^+ \) current \( I_{\text{K(M)}} \). The nature and construction of this model framework is detailed previously (22) and the model formulations are available at http://senselab.med.yale.edu/senselab/modeldb.

### Table 1. Default parameters values for the model of hippocampal CA1 pyramidal neuron used in this study.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( g_{Na} )</td>
<td>( Na^+ ) current conductance</td>
<td>35 mS/cm²</td>
</tr>
<tr>
<td>( g_{NaP} )</td>
<td>Persistent ( Na^+ ) current conductance</td>
<td>0.2 mS/cm²</td>
</tr>
<tr>
<td>( g_{K(DR)} )</td>
<td>Delayed rectifier ( K^+ ) current conductance</td>
<td>6.0 mS/cm²</td>
</tr>
<tr>
<td>( g_{K(A)} )</td>
<td>A-type ( K^+ ) current conductance</td>
<td>1.4 mS/cm²</td>
</tr>
<tr>
<td>( g_{K(M)} )</td>
<td>M-type ( K^+ ) current conductance</td>
<td>1.0 mS/cm²</td>
</tr>
<tr>
<td>( g_{Ca} )</td>
<td>( Ca^{2+} ) current conductance</td>
<td>0.08 mS/cm²</td>
</tr>
<tr>
<td>( g_{K(Cha)} )</td>
<td>( Ca^{2+} )-activated ( K^+ ) current conductance</td>
<td>10.0 mS/cm²</td>
</tr>
<tr>
<td>( g_{K(AHP)} )</td>
<td>Afterhyperpolarization-related ( K^+ ) current conductance</td>
<td>5.0 mS/cm²</td>
</tr>
</tbody>
</table>

Increasing the concentration of diclofenac not only reduced \( I_{\text{K(DR)}} \) amplitude, but also enhanced the apparent inactivation. The inhibitory effect of DIC on \( I_{\text{K(DR)}} \) can be explained by a state-dependent block where it binds to the open state of the channel according to a minimal kinetic scheme (23, 24):

\[
\begin{align*}
Closed & \xleftrightarrow{\alpha} \text{Open} & \xleftrightarrow{\beta} \text{Inactivated},
\end{align*}
\]

where \( \alpha \) and \( \beta \) are the voltage-dependent rate constant for the opening and closing of the \( Kv \) channel, \( k_{+} \) and \( k_{-} \) are those for blocking and unblocking by DIC, and \([B]\) is the DIC concentration. The unblock rate constant \( (k_{+}) \) in the presence of DIC was estimated to be 0.00432 ms⁻¹ and 0.113 ms⁻¹ μM⁻¹, respectively.

To model DIC-induced block of \( I_{\text{K(DR)}} \), an inactivation variable \( h \) was included in simulated \( I_{\text{K(DR)}} \) and the macroscopic current was then expressed as:

\[
I_{\text{K(DR)}} = g_{\text{K(DR)}} \times m(V)^4 \times h \times (V - V_{k}).
\]

Here, \( g_{\text{K(DR)}} \) is the maximal conductance of \( I_{\text{K(DR)}} \), and \( V_{k} \) the \( K^+ \) reversal potential. Because as DIC-induced block is a state-dependent process, the form of the equation describing the inactivation variables is then modified as follows:

\[
\frac{dh}{dt} = k_{+1}(1 - h) - k_{-1} \times n^4 \times h,
\]

In this study, the solution to the differential sets of ordinary differential equations in numerical simulations accompanied by studies of bifurcation diagrams was made using the simulation package XPPAUT (25, 26). Parts of numerical simulations were verified with Microsoft Excel (27). Numerical computations were generally run under a Hewlett Packard xw9300 workstation (Palo Alto, CA, U.S.A.). The conductance values used to solve the set of differential equations are summarized in Table 1.

### RESULTS

The mRNA expression for Kv3.1 in differentiated NSC-34 cells

We detected the mRNA expression of Kv3.1 (kcncl) and M-type channel (kcnq2-kcnq5) in NSC-34 cells by a semi-quantitative RT-PCR assay. Our RT-PCR analysis clearly presented the mRNA of kcncl and kcnq2-kcnq5 in NSC-34 cells (Fig. 1).

Inhibitory effect of diclofenac on \( I_{\text{K(DR)}} \) in differentiated NSC-34 cells

The whole-cell recordings of the patch-clamp technique were first used to evaluate the effect of diclofenac on ionic currents in these cells. To record \( K^+ \) outward currents and avoid
any contamination of Ca\(^{2+}\)-activated K\(^{+}\) currents, cells were bathed in Ca\(^{2+}\)-free Tyrode's solution. When the cell examined was held at \(-50\) mV and the depolarizing voltage pulses ranging from \(-80\) to \(+40\) mV in 10-mV increments were applied with a duration of 1 s, a family of outward currents accompanied by slight inactivation was elicited (Fig. 2). The threshold for elicitation of these outward currents was around \(-30\) mV, a value that is higher than that of the classical \(I_{\text{K(DR)}}\) described in the squid giant axons (28), and their current magnitudes became strongly greater with larger depolarization. This population of outwardly rectifying currents was identified as \(I_{\text{K(DR)}}\) and noted to resemble the KV3.1-encoded currents (23, 29, 30). Notably, 2 min after exposure to diclofenac (100 µM), the \(I_{\text{K(DR)}}\) amplitude was greatly reduced at the potentials between \(-20\) and \(+40\) mV. For example, when the depolarizing pulse from \(-50\) to \(+40\) mV was applied, diclofenac (100 µM) decreased the \(I_{\text{K(DR)}}\) amplitude measured at the end of voltage pulses by 53±6 % from 963±158 to 457±107 pA (n = 10). After washout of DIC, current amplitude at +40 mV was returned to 567±126 pA (n = 6). Fig. 2B illustrates the \(I-V\) relationships of \(I_{\text{K(DR)}}\) measured at the beginning and end of voltage pulses, as the data were obtained with or without addition of 100 µM DIC. To clarify whether the DIC-sensitive component of \(I_{\text{K(DR)}}\) is mediated by Kv3.1, we applied the sea anemone toxin BDS-1, which is known to be an effective inhibitor of Kv3.1 (31). BDS-1 (10 nM) can significantly suppress the amplitude of \(I_{\text{K(DR)}}\) in NSC-34 cells (data not shown). Moreover, as cells were exposed to Ca\(^{2+}\)-free Tyrode's solution containing 1 mM BAPTA, the magnitude of DIC-mediated inhibition of \(I_{\text{K(DR)}}\) in NSC-34 cells remained unaltered.

We further determined the relationship between the diclofenac concentration and the relative amplitude of \(I_{\text{K(DR)}}\). As
depicted in Fig. 3A, DIC ranging between 10 µM and 1 mM suppressed the $I_{K(DR)}$ amplitude in a concentration-dependent fashion. Half-maximal concentration required for the inhibitory effect of DIC (i.e., IC$_{50}$ value) was measured to be 73 µM, and at a concentration of 1 mM, it almost fully suppressed current amplitude. Therefore, the experimental results indicate that similar to flupirtine (15), DIC exerts a depressant action on $I_{K(DR)}$ in these cells.

Inability of naloxone, glibenclamide or apamin to reverse diclofenac-mediated inhibition of $I_{K(DR)}$ in differentiated NSC-34 cells

Earlier studies showed that diclofenac-induced analgesic action is mediated by endogenous opioids in certain nuclei of brain stem related to pain sensation (32). This compound is also thought to exert anti-nociceptive action through the activation of K$^+$ currents (3, 4). For these reasons, further experiments were performed to see whether diclofenac-mediated inhibition of $I_{K(DR)}$ observed in NSC-34 cells can be altered by subsequent application of naloxone, glibenclamide or apamin. However, as shown in Fig. 3B, neither naloxone (30 µM), glibenclamide (30 µM) nor apamin (200 nM) produced notable effects on the reduction by DIC (100 µM) of $I_{K(DR)}$ in these cells. Moreover, iberiotoxin (200 nM) or apamin (200 nM) alone produced little or no effects on the $I_{K(DR)}$ amplitude, while naloxone (30 µM) or glibenclamide (30 µM) slightly decreased $I_{K(DR)}$ amplitude by about 10%. As evidenced by these findings, it seems unlikely that DIC-mediated block of $I_{K(DR)}$ in NSC-34 cells is mediated predominantly through activation of either ATP-sensitive K$^+$ or calcium-activated K$^+$ channels.

Diclofenac-induced elevation in the inactivation rate of $I_{K(DR)}$ in differentiated NSC-34 cells

The observed $I_{K(DR)}$, during cell exposure to diclofenac tends to exhibit a pronounced peak followed by an exponential decay to a steady-state level. We next evaluated voltage and time dependence of diclofenac-induced decrease in the inactivation time constant of $I_{K(DR)}$. As illustrated in Fig. 4A, the trajectories of current inactivation with or without addition of diclofenac (100 µM) were fitted by a monoexponential function to assess
the inactivation time constants. As cells were exposed to diclofenac at a concentration of 100 µM, the inactivation time constant of $I_{K(DR)}$ elicited by membrane depolarization from –50 to +40 mV was significantly shortened to 311±21 ms (n = 8) from a control of 527±23 ms (n = 8). Furthermore, addition of diclofenac was found to accelerate the process of $I_{K(DR)}$ inactivation with a voltage-dependent property (Fig. 4B). However, during cell exposure to 100 µM DIC, the activation rate of $I_{K(DR)}$ in response to the same voltage protocol remained unaffected. No significant change in current amplitude at the holding potential of –50 mV was also demonstrated in the presence of diclofenac. Cell exposure to diclofenac did not alter the midpoint and the slope factor of $I_{K(DR)}$ inactivation curve.

Moreover, subsequent application of linopirdine (10 µM), a selective inhibitor of $I_{K(M)}$ (15, 33), did not alter diclofenac-induced increase of current inactivation process (data not shown).

**Effect of diclofenac on M-type K+ current ($I_{K(M)}$) in differentiated NSC-34 cells**

Previous work has demonstrated the ability of diclofenac to activate KCNQ2/KCNQ3 currents (7, 34). Several studies have also reported the presence of $I_{K(M)}$ in another motor-neuron-like cells, namely, NG108-15 cells (35, 36). Therefore, we further evaluated whether diclofenac produces any effects on the
amplitude of \( I_{\text{KOM}} \) expressed in NSC-34 cells. As illustrated in Fig. 5, when cells were hyperpolarized from –10 to –40 mV, the \( I_{\text{KOM}} \) amplitude were significantly greater in the presence of diclofenac at a concentration of 300 µM as compared with that in the control. In continued presence of diclofenac (300 µM), subsequent application of linopirdine (10 µM) reversed DIC-induced stimulation of \( I_{\text{KOM}} \). Similarly, addition of either flupirtine (10 µM) or meclofenamic acid (10 µM), known to be an activator of KCNQ2/Q3 channels (15, 33, 34), significantly elevated \( I_{\text{KOM}} \) amplitude (Fig. 5B). The data suggested that besides an inhibitor of \( I_{\text{KDR}} \), diclofenac is capable of stimulating \( I_{\text{KOM}} \) in differentiated NSC-34 cells.

**Effects of diclofenac on \( I_{\text{KOR}} \) and \( I_{\text{KOM}} \) in rat dorsal root ganglion neurons**

The electric properties in NSC-34 cells could be distinguishable from those in other types of neurons. We also investigated the existence of \( I_{\text{KDR}} \) and \( I_{\text{KOM}} \) in rat dorsal root ganglion (DRG) neurons. In this set of experiments, DRG neurons were bathed in Ca\(^{2+}\)-free Tyrode’s solution containing 1 µM tetrodotoxin. As shown in Fig. 6, under the same voltage profile used for NSC-34 neuronal cells, we were able to identify the properties of \( I_{\text{KOR}} \) and \( I_{\text{KOM}} \) in these cells. As cells were exposed to diclofenac (100 µM), the amplitude of \( I_{\text{KOM}} \) was increased and the \( I_{\text{KDR}} \) amplitude was diminished with a shortening of inactivation time constant. The relationships between the diclofenac concentration and the relative amplitude of \( I_{\text{KOR}} \) or \( I_{\text{KOM}} \) were constructed and are illustrated in Fig. 6C and 6D, respectively.

**Effect of varying \( g_{\text{KOR}} \) on action potential (AP) firing frequency in modeled neuron**

The next set of simulations was designed to determine the effects of \( I_{\text{KOR}} \) blockade on the frequency of action potentials firing. Fig. 7 depicts the relationship of firing frequency versus varying stimuli. As shown in Fig. 8A, as the \( g_{\text{KOR}} \) value increased from 1.0 to 5.0 mS/cm\(^2\), the firing frequency of action potentials was notably elevated. In consistent with findings described in Fig. 9 there was a notable increase in the duration of neuronal action potentials when \( g_{\text{KOR}} \) was reduced. The oscillation frequency generated from bifurcation diagram is illustrated in Fig. 8B as a function of varying \( g_{\text{KOR}} \) values. When the \( g_{\text{KOR}} \) value was lower than 10 mS/cm\(^2\), action potentials firing frequency was found to increase sharply with the increase of \( g_{\text{KOR}} \), despite the lengthening of AP width. However, as the \( g_{\text{KOR}} \) value was greater than 15 mS/cm\(^2\), the firing frequency of modeled neuron was gradually diminished with increasing \( g_{\text{KOR}} \). Therefore, worthy of being noted was that a reversal of firing frequency from positive to negative relationship emerged between the \( g_{\text{KOR}} \) values of 10 and 15 mS/cm\(^2\).

**Effect of varying \( g_{\text{KOM}} \) on the repetitive firing of simulated action potentials**

Because of the ability of diclofenac to increase \( I_{\text{KOM}} \) in NSC-34 cells, we further evaluate the possible effects of \( g_{\text{KOM}} \) on action potential firing. Fig. 10 illustrates a significant effect of \( g_{\text{KOM}} \) on the firing of simulated action potentials generated from this modeled neuron. In contrast to effects of varying \( g_{\text{KOR}} \) on action potential firing, it can be seen that as the \( g_{\text{KOM}} \) value was raised from 0 to 1.5 mS/cm\(^2\), the firing frequency of simulated APs exponentially decreased with resultant changes in the \( I_{\text{KOM}} \) amplitude (Fig. 10). When \( g_{\text{KOM}} \) was removed, action potential firing frequency was reached to be about 80 Hz, while as the \( g_{\text{KOM}} \) value was arbitrarily increased to 1.5 mS/cm\(^2\), the frequency was declined to about 10 Hz. The frequency remained relatively unaltered as \( g_{\text{KOM}} \) was higher than 1.2 mS/cm\(^2\). As the \( g_{\text{KOM}} \) value was greater than 1.6 mS/cm\(^2\), action potential firing was totally suppressed. It also needs to be mentioned that the resting membrane potential became more hyperpolarized with the increasing value of \( g_{\text{KOM}} \) (Fig. 10A). The latter results basically support the notion that \( I_{\text{KOM}} \) is a non-inactivating, voltage-dependent K\(^+\) current that activates in a time- and voltage-dependent manner at around –60 mV, which is close to the resting...
membrane potential but more negative than that for $I_{K(DR)}$ activation. Therefore, the simulation results clearly indicate that unlike $g_{K(DR)}$, appropriate changes in $g_{K(M)}$ can virtually make a significant contribution to the resting membrane potential inherent in this modeled neuron because the activation range of $I_{K(M)}$ is far more negative than that of $I_{K(DR)}$ (9, 10).

Effects of increasing $g_{K(M)}$ and decreasing $g_{K(DR)}$ on the firing of simulated action potentials

Because our experimental results made in differentiated NSC-34 neurons reflect that the depressant action of diclofenac on neuronal firing is mediated through both blockade of $I_{K(DR)}$ and activation of $I_{K(M)}$, we further evaluate how changes in $g_{K(DR)}$ and $g_{K(M)}$ influence the firing frequency of simulated APs to mimic the effect of diclofenac. As the $g_{K(M)}$ was elevated by 50% (from 1 to 1.5 mS/cm$^2$), the frequency of sustained firing as a function of $I_{stim}$ was progressively shifted in a downward direction (Fig. 11). However, to mimic the action of diclofenac on $I_{K(DR)}$ and $I_{K(M)}$, as $g_{K(M)}$ increased by 50% (from 1 to 1.5 mS/cm$^2$) with the accompanied reduction of $g_{K(DR)}$ by 50% (from 6 to 3 mS/cm$^2$), repetitive firing of simulated APs as a function of $I_{stim}$ was even more depressed. Based on simulations, it is reasonable to propose that attenuation of AP firing caused by increased $g_{K(M)}$ can be theoretically potentiated by a further reduction of $g_{K(DR)}$. In the model, the reduction of firing frequency was achieved by increasing $g_{K(M)}$ and decreasing $g_{K(DR)}$. As a corollary to these findings, the simulation results allow us to predict that modest changes in $I_{K(M)}$ and $I_{K(DR)}$ have sufficient leverage to mediate the effect of diclofenac on electrical activity in central neurons in vivo.
Effect of diclofenac on spontaneous action potentials in a modeled neuron

In order to evaluate how diclofenac alters the discharge pattern of neurons, a simulation model, originally derived from Golomb et al. (22), was implemented (Fig. 12). In this modeled cell, as $g_{K(DR)}$ was decreased from 6 to 3 mS/cm$^2$ and the block of $I_{K(DR)}$ by diclofenac at a concentration of 100 µM was simulated, the firing frequency of simulated APs was readily diminished. When $g_{K(M)}$ was further elevated to 1.5 mS/cm$^2$ in continued presence of reduced $g_{K(DR)}$ and $I_{K(DR)}$ inactivation where the DIC action was mimicked, the spontaneous APs were further reduced along with membrane hyperpolarization. As a result, the reduced $g_{K(DR)}$, the increased decay of $I_{K(DR)}$ inactivation and the elevated $g_{K(M)}$, which mimics the DIC action, can combine to cause changes in the firing of simulated APs.

DISCUSSION

In our study, diclofenac has a complex and interesting profile in that it decreases action potentials firing via mechanisms other than an interaction at the M-type K$^+$ channels. One of these mechanisms may involve $I_{K(DR)}$ (i.e., fast delayed rectifier), the biophysical properties of which are distinguishable from the Hodgkin-Huxley-type delayed rectifier current (23, 28, 36). In addition to a stimulatory effect of $I_{K(M)}$, diclofenac suppressed $I_{K(DR)}$ in a concentration- and time-dependent fashion. Given that the importance of $I_{K(DR)}$ (i.e., Kv3.1-encoded current) in contributing to neuronal excitability and automaticity, it is conceivable that diclofenac-induced block of $I_{K(DR)}$ is involved in the alteration of neuronal firing, particularly at high-spiking neurons. We have also integrated experimental and theoretical approaches in order to study potential influence of this agent or its functionally related compounds on neuronal firing. The computer simulation with minimal binding scheme suggests that diclofenac may act as a state-dependent blocker of Kv channel.

The biophysical nature of $I_{K(DR)}$ in NSC-34 cells resembles the Kv3.1-encoded current because of positive mRNA detection of Kv3.1 and a great sensitivity to inhibition by BDS-1. NSC-34 cells differentiated with 1% low serum medium for 48 hours were found to increase the mRNA level of class III β-tubulin, a neuron-specific marker, as compared with that in normal cells. The Kv channels from the Kv3.1 type, which are the major determinant of $I_{K(DR)}$ in NSC-34 cells, another motor neuron-like cells (NG108-15), and DRG neurons (20), are thought to make little contribution to the resting membrane potential. However, due to their fast activating and deactivating properties, these channels are responsible for...
spike repolarization and after-hyperpolarization of neurons with high-frequency firing (24, 37). The inhibition of $I_{K(DR)}$ caused by diclofenac is able to retard the AP repolarization and slow recovery of $I_{Na}$ inactivation, which may pose the neuron to decreased rhythmic firing of APs. However, Kv3.1 channels are well known to be regulated at both the transcriptional and posttranslational levels. When $I_{K(DR)}$ amplitude is considerably high, this effect may diminish neuronal hypo-excitability of $I_{K(M)}$ activation as predicted from the modeled neuron, while with a lower value of $g_{K(DR)}$ it can facilitate the inhibitory effects of $I_{K(M)}$ activation on AP firing.

Based on our studies, it is indeed conceivable that the effects of Kv7-channel openers on AP firing rely on the pre-existing magnitude of $I_{K(DR)}$, despite no clear evidence to show that KV3 and Kv7 channels can co-assemble to form a protein complex. The physiological importance of Kv7 channels has been recognized in a variety of brain regions including the hippocampus, neocortex and cerebellar cortex, where are key sites for neuronal network oscillations (9-11). The effect of diclofenac observed in NSC-34 neurons was noted to display biophysical or pharmacological features that were not merely confined to stimulation of $I_{K(M)}$. The $IC_{50}$ value for the effect of this agent on $I_{K(DR)}$ was about 73 µM, a value that is close to the concentration required for its stimulatory action on Kv7 channels. Its effects on $I_{K(DR)}$ are most likely to be therapeutically or clinically relevant. Our study thus prompted us to propose that Kv3 and Kv7 channels have a considerable pharmacological overlap. It is tempting to speculate that diclofenac can exert an interaction at a similar binding site inherently existing on KV3 and Kv7 channels.

It has been previously demonstrated that central analgesic action of diclofenac tends to be mediated by the release of endogenous opioids in certain brain stem nuclei which is linked to control of pain sensation (32). However, in this study, subsequent application of naloxone, a blocker of opioid receptors, did not reverse inhibition of $I_{K(DR)}$ caused by diclofenac. Moreover, when NSC-34 cells were further exposed to naloxone, diclofenac-induced widening of AP remained unaltered. Therefore, findings from our results reflect that the action of diclofenac on ionic currents observed in NSC-34 cells does not arise from the release of opioid-like substance(s) or the binding of this agent to opioid receptors.

Interestingly, it has been proposed that phospholipase C- and Ca$^{2+}$/phosphatidylinositol 4,5-bisphosphate-mediated inhibition of $I_{K(M)}$ in sensory neurons may represent one of the general mechanisms underlying pain produced by inflammatory mediators (38). Our study suggests the potential $I_{K(M)}$-modulating role of diclofenac on pain disorders which are produced by inflammatory mediators, in addition to its traditionally anticipated role as an anti-inflammatory agent.

As the $I_{stim}$ value is elevated, the frequency is increased. Notably, for mimicking the action of diclofenac on neuronal firing, the combination of reduced $g_{K(DR)}$ and increased $g_{K(M)}$ (c) depresses the relationship of oscillation frequency versus $I_{stim}$ in a downward direction to a greater extent as compared with that under the increase of $g_{K(M)}$ alone (b).

**Fig. 11.** Firing frequency versus $I_{stim}$ relationships of modeled neuron with different values of $g_{K(DR)}$ and $g_{K(M)}$. Bifurcation diagrams of frequency as a function of varying $I_{stim}$ were derived. (a): $g_{K(DR)}=6.0$ mS/cm$^2$, $g_{K(M)}=1.0$ mS/cm$^2$; (b): $g_{K(DR)}=6.0$ mS/cm$^2$, $g_{K(M)}=1.5$ mS/cm$^2$; (c): $g_{K(DR)}=3.0$ mS/cm$^2$, $g_{K(M)}=1.5$ mS/cm$^2$. As the $I_{stim}$ value is elevated, the frequency is increased. Notably, for mimicking the action of diclofenac on neuronal firing, the combination of reduced $g_{K(DR)}$ and increased $g_{K(M)}$ (c) depresses the relationship of oscillation frequency versus $I_{stim}$ in a downward direction to a greater extent as compared with that under the increase of $g_{K(M)}$ alone (b).

**Fig. 12.** Simulation modeling used to mimic diclofenac effects on repetitive firing of neuronal action potentials. The model was developed based on the electrophysiological properties of hypothalamic CA1 pyramidal cells as described in materials and methods. In (A), the firing of spontaneous APs under control condition (i.e., $g_{K(DR)}=6$ mS/cm$^2$ and $g_{K(M)}=1$ mS/cm$^2$). In (B), when $g_{K(DR)}$ was decreased from 6 to 3 mS/cm$^2$ and the diclofenac concentration was set at 100 µM, the AP firing was diminished together with membrane hyperpolarization. In (C), when $g_{K(M)}$ was elevated 1.5 mS/cm$^2$ in the continued presence to reduced $g_{K(DR)}$ and increased inactivation rate of $I_{K(DR)}$, where the diclofenac (100 µM) action was mimicked, the spike discharge was further decreased.

---

**Fig. 11.** Firing frequency versus $I_{stim}$ relationships of modeled neuron with different values of $g_{K(DR)}$ and $g_{K(M)}$. Bifurcation diagrams of frequency as a function of varying $I_{stim}$ were derived. (a): $g_{K(DR)}=6.0$ mS/cm$^2$, $g_{K(M)}=1.0$ mS/cm$^2$; (b): $g_{K(DR)}=6.0$ mS/cm$^2$, $g_{K(M)}=1.5$ mS/cm$^2$; (c): $g_{K(DR)}=3.0$ mS/cm$^2$, $g_{K(M)}=1.5$ mS/cm$^2$. As the $I_{stim}$ value is elevated, the frequency is increased. Notably, for mimicking the action of diclofenac on neuronal firing, the combination of reduced $g_{K(DR)}$ and increased $g_{K(M)}$ (c) depresses the relationship of oscillation frequency versus $I_{stim}$ in a downward direction to a greater extent as compared with that under the increase of $g_{K(M)}$ alone (b).

**Fig. 12.** Simulation modeling used to mimic diclofenac effects on repetitive firing of neuronal action potentials. The model was developed based on the electrophysiological properties of hypothalamic CA1 pyramidal cells as described in materials and methods. In (A), the firing of spontaneous APs under control condition (i.e., $g_{K(DR)}=6$ mS/cm$^2$ and $g_{K(M)}=1$ mS/cm$^2$). In (B), when $g_{K(DR)}$ was decreased from 6 to 3 mS/cm$^2$ and the diclofenac concentration was set at 100 µM, the AP firing was diminished together with membrane hyperpolarization. In (C), when $g_{K(M)}$ was elevated 1.5 mS/cm$^2$ in the continued presence to reduced $g_{K(DR)}$ and increased inactivation rate of $I_{K(DR)}$, where the diclofenac (100 µM) action was mimicked, the spike discharge was further decreased.
to accelerate the process of current inactivation in NSC-34 neurons (data not shown). The diclofenac-mediated reduction of \( I_{\text{KDR}} \) in combination with attenuation of AP firing may be accentuated in continued presence of benzodiazepine agonists. Therefore, it is important to evaluate the extent to what \( I_{\text{KDR}} \) blockade induced by these agents with concomitant activation of \( I_{\text{KOM}} \) contributes to the anticonvulsant activity of benzodiazepine agonists (39), as these latter agonists exert inhibitory effects on \( I_{\text{KDR}} \) (40).

An intriguing consequence of our simulation findings is that the inhibitory effects of diclofenac on neuronal firing in vivo may result from activation of \( I_{\text{KOM}} \) and inhibition of \( I_{\text{KDR}} \), especially during the lower value of \( g_{\text{KOM}} \). It is clear that decreasing \( g_{\text{KOM}} \) can facilitate whereas increasing \( g_{\text{KOM}} \) counteracts the inhibition of oscillating frequency caused by increasing \( g_{\text{KDR}} \). The main reason for these results can be explained by the fact that the recovery of \( Na^+ \) channels was hindered by decreased \( g_{\text{KOM}} \) (28, 37), while the inactivation of \( Na^+ \) channels was largely enhanced by the increase of \( g_{\text{KOM}} \) (22).

Activation of \( I_{\text{KOM}} \) is so fast as to influence the repolarization of single AP. Although decreased \( g_{\text{KOM}} \) can prolong the duration of spike after depolarization and may subsequently facilitate to its escalation to a burst, the concomitant increase of \( g_{\text{KOM}} \) as numerically simulated here for the diclofenac action will consequently render neuronal hyperexcitability to be suppressed.

A recent study found rebamipide could attenuate NSAIDs including diclofenac’s inducing lipid peroxidation in gastric epithelial cells by increasing the expression of manganese superoxide dismutase protein and decreasing superoxide anion leakage from mitochondria in both gastric and small intestinal epithelial cells. Whether this effect is related to the ionic mechanism described in our study is worth further studies (41).

In summary, diclofenac can directly block \( I_{\text{KDR}} \) in a concentration-dependent manner and accelerate the decay of \( I_{\text{KDR}} \) in differentiated NSC-34 and DRG neurons. Diclofenac-mediated reduction of neuronal AP firing might not be solely explained by the activation of \( I_{\text{KOM}} \) although one frequently cited explanation for the beneficial effect of this and other functionally similar compounds is that they are efficacious as an activator of \( I_{\text{KOM}} \) (7, 8, 12, 14, 33, 42, 43). Because \( I_{\text{KOM}} \) and \( I_{\text{KDR}} \) tend to have reciprocal effects, it is difficult to experimentally determine their relative importance for the effects of these agents on action potential firing (44), including their subtypes (45). However, based on our simulations, it needs to speculate that, in terms of action potential firing, a combined approach using the activation of \( I_{\text{KOM}} \) and the inhibition of \( I_{\text{KDR}} \) would be more useful than using the activator of \( I_{\text{KOM}} \) alone. Suppression of sustained firing by these agents may similarly involve both activation of \( I_{\text{KOM}} \) and inhibition of \( I_{\text{KDR}} \). The contribution of \( I_{\text{KOM}} \) to the pattern and frequency of action potential firing may be accentuated under certain conditions in vivo as a result of either high expression of Kv3 channels or pharmacological modulation of Kv3 channels (e.g., diazepam or midazolam). If similar findings are experimentally made in neurons occurring in cell culture or in vivo to those described herein, the actions of DIC or its functionally related agents (e.g., flurpirine) will result in significant changes in neuronal excitability (12).

Acknowledgements: The work in this laboratory was supported by grants from the National Science Council (NSC-101-2320-B-006-009, NSC-100-2314-B-006-002, and NSC-101-2314-B-006-059), Taiwan, and from the Aim for the Top University Project, National Cheng Kung University, Taiwan. The authors acknowledge Hsien-Chin Huang who assisted in cell preparations used for this study.

Conflict of interests: None declared.

REFERENCES

7. Takahashi Y, Kaba H. Muscarinic receptor type 1 (M1) stimulation, probably through KCNQ/Kv7 channel closure, increases spontaneous GABA release at the dendrodendritic synapse in the mouse accessory olfactory bulb. Brain Res 2010; 1339: 26-40.
280


Received: February 4, 2013
Accepted: June 27, 2013

Author's address: Dr. Sheng-Nan Wu, Department of Physiology, National Cheng Kung University Medical College, No. 1, University Road, Tainan 70101, Taiwan.
E-mail: snwu@mail.ncku.edu.tw