Inhibition of Delayed Rectifier Potassium Channels and Induction of Arrhythmia

A NOVEL EFFECT OF CELECOXIB AND THE MECHANISM UNDERLYING IT

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Selective inhibitors of cyclooxygenase-2 (COX-2), such as rofecoxib (Vioxx), celecoxib (Celebrex), and valdecoxib (Bextra), have been developed for treating arthritis and other musculoskeletal complaints. Selective inhibition of COX-2 over COX-1 results in preferential decrease in prostacyclin production over thromboxane A2 production, thus leading to less gastric effects than those seen with nonselective COX inhibitors such as acetylsalicylic acid (aspirin). Here we show a novel effect of celecoxib via a mechanism that is independent of COX-2 inhibition. The drug inhibited the delayed rectifier (Kv2) potassium channels from Drosophila, rats, and humans and led to pronounced arrhythmia in Drosophila heart and arrhythmic beating of rat heart cells in culture. These effects occurred despite the genomic absence of cyclooxygenases in Drosophila and the failure of acetylsalicylic acid, a potent inhibitor of both COX-1 and COX-2, to inhibit rat Kv2.1 channels. A genetically null mutant of Drosophila Shab (Kv2) channels reproduced the cardiac effect of celecoxib, and the drug was unable to further enhance the effect of the mutation. These observations reveal an unanticipated effect of celecoxib on Drosophila hearts and on heart cells from rats, implicating the inhibition of Kv2 channels as the mechanism underlying this effect.

Selective COX-2 inhibitors, or coxibs, were developed for use as nonsteroidal anti-inflammatory drugs without adverse gastric effects (1, 2). Gastric effects of nonselective cyclooxygenase inhibitors, such as acetylsalicylic acid (aspirin), arise in main part from inhibition of cyclooxygenase-1. A selective inhibition of COX-2 by coxibs helps avoid these effects (3, 4); however, adverse cardiovascular effects complicate the use of coxibs (5–7). These effects of coxibs have been attributed to a reduction in antithrombotic, COX-2-derived prostacyclin without a reduction in prothrombotic, COX-1-derived platelet thromboxane. This shifts the balance toward a prothrombotic state resulting in clot formation (7).

The adverse effects of drugs like celecoxib arising from their selective inhibition of COX-2 is currently the topic of intense discussion (7, 8). We now show that celecoxib can have additional, heretofore unanticipated effects on Drosophila hearts and rat heart cells via a pathway independent of cyclooxygenase inhibition. At low micromolar concentrations, celecoxib reduced heart rate and induced pronounced arrhythmia in Drosophila hearts. A similar effect was observed in rat cardiomyocytes in culture in which the drug reduced the beating rate in clusters of cells and made the beating arrhythmic. These effects were not mediated via cyclooxygenase inhibition but involved inhibition of the voltage-activated delayed rectifier K⁺ channels (Kv2) by the drug. These observations reveal a new molecular mechanism underlying the effects of celecoxib that may operate in addition to its prothrombotic influence. The data also raise the possibility of effects on other tissues that express Kv2 channels.

EXPERIMENTAL PROCEDURES

Materials—Quinidine, 4-aminopyridine acetylsalicylic acid, and other chemicals were purchased from Sigma. Celebrex capsules were used as the source of celecoxib so as to examine the effect of the actual formulation used in clinical settings (9). Celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide) from Celebrex capsules containing 200 mg of the drug was dissolved in dimethyl sulfoxide (Me₂SO). Additional details are given under supplemental “Experimental Procedures.”

Drosophila Heart Preparation—Strains of Drosophila melanogaster were maintained at 21 °C. Activity of the heart was monitored at 21 °C using a larval preparation described previously (10). Heart rate (HR) was counted for 6 min before drug application (the basal rate), for 10 min in the presence of the drug, and for 10 min after washing out the drug. Video recordings of heart activity were made at a resolution of 640 × 480 pixels at 30 frames/s. Analysis of video data is described under supplemental “Experimental Procedures.”

Rat Ventricular Cardiomyocytes—Rat fetuses (stage G20, 1 day before birth) from timed-pregnant Holtzman rats were kindly donated by Dr. J. Olson. The protocols used were approved by the Institutional Animal Care and Use Committee of the State University of New York at Buffalo. Ventricular myocytes were isolated and cultured under aseptic conditions. The cardiomyocyte cultures were used in experiments 24–48 h.
after seeding the cells when cells were beating rhythmically. Beating activity in cardiomyocytes was monitored at 37 °C. The activity was video-recorded for 2 min each before drug application (basal rate), at the end of 45-min exposure to the drug, and 15 min after wash-out. Additional details and analysis of video data are given under supplemental “Experimental Procedures.”

Recordings from Drosophila K^+ Channels—Delayed rectifier K^+ current (I_{KS}) and the fast transient K^+ current (I_A) were recorded from the larval body wall muscles using two-microelectrode voltage clamping. Recordings were made from muscle fiber 12 (11). Currents were elicited by 500-ms voltage steps from a holding potential of −80 mV. I_{KS} was measured as current at the end of the 500-ms testing pulse, and I_A was measured as the initial peak current. A dose-response curve for I_{KS} (see Fig. 4E) was generated by examining the current for voltage steps to +40 mV. For more detailed current-voltage relationships (see Fig. 4, A and C), membrane potential was stepped to between −40 and +40 mV in 10-mV increments. The currents were divided by cell capacitance to obtain current densities. For more details, see supplemental “Experimental Procedures.”

Recordings from Rat and Human Kv2.1 Channels Expressed in Human Embryonic Kidney (HEK)-293 Cells—For rat channels, the pcDNA-Kv2.1 vector, provided by Dr. H. Y. Gaisano of the University of Toronto (12, 13), was expressed in HEK-293 cells. After establishing the whole-cell configuration, the cell was held at −70 mV, and 500-ms test pulses were applied in 10-mV increments to potentials up to +40 mV. Drugs were applied using a fast perfusion system (ValveLink 8, AutoMate Scientific). Recordings from rat Kv2.1 channels were made in the presence of the drug were normalized to the currents obtained before the drug exposure. For more details, see supplemental “Experimental Procedures.”

RESULTS

We examined whether celecoxib affected cardiac rhythm in Drosophila hearts and rat cardiomyocytes. We examined this first in Drosophila, as Drosophila serves as an important model for human cardiac pharmacology and cardiomyopathies (10, 15–18) as well as other human disorders (19, 20). Heart rate in Drosophila was significantly reduced by exposure to 3 μM celecoxib (Fig. 1, A and B). The heart stopped beating within a minute of applying 30 μM and within seconds of exposure to 100 μM celecoxib. The dose-response curve showed an EC_{50} (50% reduction in HR) of 11.2 μM (Fig. 1C). Me_{2}SO, used for dissolving the drug, did not affect HR at 0.5%, the highest concentration used in these experiments (Fig. 1, A and B).

In addition to decreasing HR, celecoxib affected cardiac rhythm. Each trace in Fig. 2A shows HR from one animal as it varied over time with drug application. Although HR fluctuations in a pool of samples averaged out (Fig. 1A), they were observed in individual animals even at low concentrations (Fig. 1B, C).

FIGURE 1. Effects of celecoxib on Drosophila heart rate. A, average HR is shown for 5–16 larvae as specified, before drug application, in the presence of celecoxib (horizontal bars), and after the wash-out. B, a bar plot summarizing the effect of celecoxib on heart rate is shown. Treatment with 0.5% Me_{2}SO, the maximum concentration of Me_{2}SO used with celecoxib, did not change the HR significantly. A significant effect was seen with celecoxib at 3 μM (p < 0.05, analysis of variance) and higher concentrations (p < 0.001); the inhibition of HR was partially reversed upon washing out the drug. 1, HR before drug application; 2, HR in the presence of celecoxib; 3, HR after wash-out. C, a dose-response curve for the effect of celecoxib on heart rate is shown. The curve represents a fit to Hill equation. Drop lines show EC_{50}. In this and the following figures, error bars show ± S.E. n, number of experiments; *, p < 0.05; **, p < 0.001.
Although 1 μM celecoxib did not affect rhythm in all cases (Fig. 2B), it induced intermittent arrhythmia in 5 of 17 cases (Fig. 2C). Similarly, 3 μM celecoxib induced pronounced intermittent arrhythmia (Fig. 2, A and D) with several minutes of arrhythmic activity followed by several minutes of relatively regular heartbeat. Arrhythmia became so severe at 10 μM that in 4 of 16 animals, heartbeat was occasionally punctuated by tempo-
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FIGURE 2. Effects of celecoxib on Drosophila cardiac rhythm. A, celecoxib introduced a pronounced variability in interbeat intervals as well as in the rhythm and the amplitude of contractions (Fig. 2C). Similarly, 3 μM celecoxib induced arrhythmic variability in HR, shown here for individual animals as percentage of the HR before drug application. B–E, traces represent the relative displacement of an edge of the larval heart. Video recordings of heart wall movements were obtained immediately before application of celecoxib (basal), at 8–10 min after wash-in of celecoxib, and at 8–10 min after washing out the drug. In most cases 1 μM celecoxib (B) slightly reduced HR, but in 5 of 17 cases it also induced a severe transient arrhythmia (C). D, 3 μM celecoxib reduced HR and induced arrhythmia. E, in addition to triggering severe arrhythmia in most cases, 10 μM celecoxib induced transient heart arrest in 4 of 16 larvae (data shown on a compressed scale). F, to assess drug-induced irregularity in HR, coefficient of variation (CV) was used as explained in supplemental Fig. 1. Celecoxib significantly increased irregularity of HR at 1 μM and higher concentrations (analysis of variance), *p < 0.05.

To examine the effect of celecoxib on mammalian heart, we used cultured cardiomyocytes from rat fetuses at stage G20 (1 day before birth). These cells can establish a spontaneous beating rhythm in culture and provide a model for cardiac function in humans (21–25). Celecoxib reduced the rate of beating in cardiomyocytes with significant effect occurring at 3 μM and with an EC50 of 7.7 μM (Fig. 3). This compares with an EC50 of 11.2 μM for HR decrease in Drosophila. The drug also induced variability in the beat. As in the case of Drosophila, this irregularity was more marked in individual samples (Fig. 3B) and was averaged out in a pool of samples (Fig. 3A). Celecoxib significantly increased the coefficient of variation in rhythmicity (Fig. 3D). Thus celecoxib affected rat cardiomyocytes and Drosophila heart in a similar way.

Although the prothrombotic effects of coxibs have been attributed to selective cyclooxygenase-2 inhibition, there is no report on the presence of cyclooxygenases in Drosophila. We probed the existence of relevant sequences in the genomic and the proteomic data bases of Drosophila. COX is composed of three units: an epidermal growth factor-like domain, a membrane-binding motif, and a domain with two catalytic sites, one for cyclooxygenase activity and one for hydroperoxidase reaction (26, 27). Nucleotide-nucleotide, protein-nucleotide, and protein-protein BLASTs in D. melanogaster data bases using human COX-2 sequence elicited some similarity with genes and proteins characterized by peroxydase activity or an epidermal growth factor-like domain but did not yield a similarity with respect to COX-2 activity. Amino acids 510–535 of the COX-1 and COX-2 sequences, constituting the cyclooxygenase active site, are highly conserved among species (supplemental Table 1) (27). However, BLAST searches using human active site sequences found no similarity in Drosophila. Also, Ser-530, a residue irreversibly acetylated by acetylsalicylic acid, and the surrounding amino acids are virtually identical in all species for both isoforms of COX (27, 28) but were not detected in BLAST searches in Drosophila. Thus the effects reported above in Drosophila may not occur via inhibition of COX-2.

Because K+ channels play a significant role in maintaining the integrity of the cardiac rhythm (29–34), we examined possible effects of celecoxib on K+ currents in Drosophila muscles. The delayed rectifier K+ current in Drosophila (IKS), carried by Kv2 (Shab) channels (35–37), was blocked by celecoxib at micromolar concentrations (Fig. 4, A and C). IKS elicited at +40 mV (Fig. 4D) showed an effect in a similar concentration range as the effect on heartbeat. Celecoxib inhibited IKS with IC50 of 10.3 μM with significant inhibition starting at 1 μM (Fig. 4E). This compares with an EC50 of 11.2 μM for reduction in HR. Furthermore, IKS showed very little inactivation in the absence of the drug, but celecoxib increased the rate of inactivation significantly (Fig. 4D).

Celecoxib did not inhibit the remaining two voltage-activated K+ currents in the muscles: IA, carried by the Shaker channels (37, 38), and IKP, carried by as yet unidentified channels (39). The drug did not inhibit IA at 20 μM (Fig. 4, B and C), a concentration 20-fold higher than the concentration that significantly affected IKS (Fig. 4E) and cardiac rhythm (Fig. 2F). Similarly, celecoxib did not inhibit IKP at 20 μM (data not shown).
Mammalian Kv2.1 channels are ortholog of Drosophila Shab (Kv2) channels and play a significant role in cardiac function (40–45). We examined the effect of celecoxib on rat Kv2.1 channels expressed in HEK-293 cells (12). Celecoxib inhibited Kv2.1 current in a similar range of concentrations as in the case of $I_{KS}$ (Fig. 5, A and D). An $IC_{50}$ of 10.3 μM for Kv2.1 (Fig. 5D) compares with an $IC_{50}$ of 10.3 μM for Drosophila $I_{KS}$ and an $EC_{50}$ of 7.7 μM for reduction of rate of beating in cardiomyocytes. As in the case of $I_{KS}$, celecoxib made inactivation faster for rat Kv2.1 channels (Fig. 5C). The current started recovering from the effect of low drug concentrations within a minute, and it showed less recovery during this time frame with increasing concentrations of the drug (Fig. 5E).

To confirm whether inhibition of rat Kv2.1 current occurred independent of COX-2 inhibition by celecoxib, we used acetylsalicylic acid, which inhibits both COX-1 and COX-2. The current was not reduced by 1 mM acetylsalicylic acid (Fig. 5B), a concentration that is more than 400-fold higher than the IC$_{50}$ of 2.4 μM for COX-2 inhibition by acetylsalicylic acid (46). Thus inhibition of COX-2 could not be mediating the effect of celecoxib on rat Kv2.1 channels.

To further examine the link between the effect of celecoxib on channels and its effect on heartbeat, we used Drosophila, with which we could combine the strength of pharmacology with that of available genetic tools. Quinidine blocks Drosophila Shab channels in a highly selective manner (47–49). If celecoxib induced arrhythmia by inhibiting these channels, then quinidine also would induce arrhythmia. Previous studies have shown reduction of HR in Drosophila by quinidine without examining its effect on the rhythm (10). In these previous studies, quinidine reduced HR with an IC$_{50}$ of 79 μM. In the current experiments, 30 μM quinidine induced severe irregularity in heartbeat (Fig. 6, A and B), again implicating Shab channels in this phenomenon.

Although quinidine shows high selectivity for Shab channels in Drosophila (47), its effect on other channels cannot be completely ruled out. A genetic approach using a mutation in the Shab gene was pursued to corroborate further the link between Shab channel disruption and arrhythmia. The $Shab^{B}$ mutation is predicted to eliminate the Shab channels (36). Heartbeat in
Shab \(^7\) larvae showed a severe effect with full contractions of the heart interspersed between partial contractile events similar to fibrillation-type activity. In addition, the rate and pattern of contractions varied from larva to larva as well as at different times within the same larva. Fig. 6A shows two such recordings from Shab \(^7\) mutants. Although HR in terms of full contractions appeared to be substantially reduced in Shab \(^7\) (Fig. 6A), it was difficult to quantify HR because of many partial contractions with different amplitudes. Variability in heart rhythm in Shab \(^7\) was compared with that in the wild type. As shown in Fig. 6B, Shab \(^3\) animals exhibited a strongly arrhythmic heartbeat.

If Shab channels are involved in mediating the action of celecoxib on cardiac rhythm, then the drug may not be able to further augment the effect of the Shab \(^7\) mutation as the mutation has already disrupted these channels. 10 \(\mu\)M celecoxib, which itself produced significant arrhythmia, was unable to augment further the arrhythmic effect of the mutation (Fig. 6C). This strongly supports the idea that the arrhythmic effect of celecoxib occurred at least in major part via the Shab channels.

As a preliminary step toward exploring the relevance to humans of the above observations, we examined whether celecoxib had any effect on human Kv2.1 channels. There is a strong overlap among Drosophila Shab, rat Kv2.1, and human Kv2.1 channels from molecular, pharmacological, and physiological points of view. For example, rat and human Kv2.1 channels have identical amino acid sequences over the membrane-spanning region (50). At a concentration of 3 \(\mu\)M, celecoxib strongly inhibited the current through human Kv2.1 channels expressed in HEK-293 cells (Fig. 7). In addition, as in the case of Drosophila Shab and rat Kv2.1 channels, the drug increased the rate of inactivation in human Kv2.1 channels.

**DISCUSSION**

Until now, the adverse effects of celecoxib have been attributed to a single mechanism-reduction in COX-2-derived prostacyclin but not in COX-1-derived platelet thromboxane resulting in clot formation in response to a perturbation (Fig. 8) (51–53). The data presented here reveal an important new effect of celecoxib. At low micromolar concentrations, the drug reduced heart rate in Drosophila and induced pronounced arrhythmia. In a similar fashion, it reduced the rate of beating in rat heart cells in culture and made the beating arrhythmic.

The above effects were mediated via an unanticipated mechanism, i.e. through inhibition of delayed rectifier potassium channels (Fig. 8) rather than through inhibition of cyclooxygenases as Drosophila lacks these enzymes. The failure of acetylsalicylic acid to inhibit rat Kv2.1 channels supports the COX-independence of the channel inhibition by celecoxib. The role of the channels in mediating the cardiac effect in Drosophila
likely cardiac implications of celecoxib therapy in humans. In addition, Kv2.1 channels play an important physiological role in several tissues such as pancreas, pulmonary arteries, placental vasculature, and hippocampal and cortical pyramidal neurons (56–58). An effect of celecoxib on Kv2.1 channels in these tissues could likely have an impact on the functioning of the tissues. However, any speculation on likely human implications of the observations reported here would be premature. The extent to which human cardiac and other functions may be altered cannot be gauged at this stage because of likely differences among Drosophila, rats, and humans in profiles of expression of various ion channels and accessory subunits and in the role of these channels in action potentials and contractility. In addition, any effect in a particular tissue will depend on the concentration of the drug in that tissue. Celecoxib concentration that shows significant Kv2.1 inhibition (1 μM) is in the range of plasma concentration achieved at therapeutic dosages in humans. For example, the peak drug concentration in human plasma after the minimal adult therapeutic dose of 200 mg is generally between 1.7 and 2.8 μM and may be as high as 6.2 μM in women aged 65 or older (9). However, available drug concentration in specific tissues is not known, and drug binding to plasma proteins on one hand and a large volume of distribution on the other (9) make it difficult to assess drug concentration in specific tissues. For all these reasons, the effects reported here cannot be extrapolated to human heart or other tissues without further experiments to explore directly such possibilities. The data presented here make a case for pursuing such experiments and for examining whether the mechanistic direction described here for the effects of celecoxib has physiological and clinical relevance in humans.

The data presented here do not allow for an extrapolation to other selective COX-2 inhibitors because the observed effect did not depend on COX inhibition. Although the previously described cardiovascular complications arising from the prothrombotic effects of coxibs have been seen to a varying degree in the case of most coxibs, the effect described here may or may not translate to other coxibs depending on the nature of the interacting domains between the channel and the drug molecule. As a fortuitous outcome of the above observations, lack of cyclooxygenases in Drosophila makes it valuable for investigating side effects of COX-2 inhibitors and other nonsteroidal anti-inflammatory drugs that occur via pathways possibly independent of COX inhibition.

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