Published in final edited form as:

Neuroscience. 2008 July 17; 154(4): 1525–1532. doi:10.1016/j.neuroscience.2008.05.004.

EFFECTS OF CELECOXIB ON IONIC CURRENTS AND SPONTANEOUS FIRING IN RAT RETINAL NEURONS

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Abstract

Accumulating evidence suggests that the side effects of celecoxib, widely used to treat muscle and joint pain, may be mediated in part through cyclooxygenase-2 (COX-2) independent mechanisms, such as inhibition of ion channels. In this study we report effects of celecoxib on ionic currents and neuronal activity in isolated rat retinal neurons. We found that celecoxib suppressed voltage-gated potassium currents in retinal bipolar cells with an effective concentration to inhibit 50% of function (EC₅₀) of 5.5 μ M. In retinal amacrine and ganglion cells, celecoxib inhibited voltage-dependent sodium channels with an EC₅₀ of 5.2 μ M, and voltage-dependent transient and sustained potassium currents with EC₅₀s of 16.3 and 9.1 μ M, respectively. Notably, the rate of spontaneous spike activity was dramatically suppressed in ganglion and amacrine cells with an EC₅₀ of 0.76 μ M. All actions of celecoxib on ionic currents and action potentials occurred from the extracellular side and were completely reversible. These findings indicate that inhibition of ion channels by celecoxib in the CNS may affect neuronal function at clinically relevant concentrations.

Keywords

potassium channels; sodium channels; voltage-gated channels; electrophysiology; coxibs; pharmacology

Selective cyclooxygenase-2 (COX-2) inhibitors, or coxibs, were developed for use as non-steroidal anti-inflammatory drugs (NSAIDs) without adverse gastric effects (Kalgutkar et al., 1998; Flower, 2003). However, the use of coxibs has been complicated by reputed adverse cardiovascular effects (Graham et al., 2005). Although still controversial, increased cardiovascular risks may also be associated with celecoxib (Solomon et al., 2005; Arber et al., 2006) and other coxibs (Mitchell and Warner, 2006).

Celecoxib's mechanism of action is inhibition of cyclooxygenases, but evidence has emerged that it can target other enzymatic and cellular mechanisms also. For instance, celecoxib inhibits carbonic anhydrases with nanomolar affinity (Weber et al., 2004; Dogne et al., 2007) and exhibits toxicity toward neonatal rat cardiac myocytes (Hasinoff et al., 2007). Celecoxib can induce apoptosis and inhibit cell cycle progression at micromolar concentrations (Grosch et al., 2006; Kern et al., 2006). We have recently shown that celecoxib can reduce heart rate and induce arrhythmia in *Drosophila*, which lack cyclooxygenase genes, and affect rate and

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regularity of beating in cultured rat ventricular cardiomyocytes. These effects are linked to inhibition of the Kv2 potassium channels (Frolov et al., 2008). In neurons, low micromolar concentrations of celecoxib inhibit voltage-activated sodium channels (Park et al., 2007) and L-type voltage-gated calcium channels (Zhang et al., 2007). However, the effect of celecoxib on neural activity has not been investigated. There have been sporadic reports that celecoxib might affect vision (Coulter et al., 2003; Fraunfelder et al., 2006), which prompted us to explore its action in retina.

In this study we investigated the effects of celecoxib on ionic currents and spontaneous spike activity in dissociated rat retinal neurons. We found that celecoxib reversibly inhibited potassium currents in bipolar cells, non-spiking retinal interneurons. Celecoxib also inhibited sodium and potassium currents in spiking third order retinal neurons. Within the putative range of human therapeutic concentrations, celecoxib reduced the rate of spontaneous firing in these retinal neurons.

EXPERIMENTAL PROCEDURES

Cell culture

All animal procedures were carried out in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University at Buffalo. Care was taken to minimize the number of animals used and their suffering. The retinal cells were dissociated as described by Luo et al. (2004). Briefly, immature (P13-25) Sprague–Dawley rats were anesthetized with pentobarbital, decapitated, and the eyes enucleated. The retinas were removed, minced and washed in Ca^{2+} and Mg^{2+} -free Ringer's solution supplemented with 0.1 mM EDTA. The tissue fragments then were incubated in 0.2% pre-activated papain for 20 min at 37 °C (Luo et al., 2004). Individual cells were dissociated by trituration and washing in NeuroBasal A medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2% B27 additive and 2% fetal bovine serum. Cells were seeded into poly-L-lysine-coated culture dishes and stored in a 5% CO_2 incubator maintained at 37 °C. Experiments were performed 1–3 days after plating.

Extraction of celecoxib

Fifteen 200-mg capsules of commercially available celecoxib ((4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzene-sulfonamide)) were disassembled and the contents were mixed as a suspension in 50 mL of HPLC-grade methanol. The mixture was stirred for 15 h, filtered through a small pad of Celite, and the filter cake was washed with 5 mL of methanol. The combined filtrates were concentrated and the residue was recrystallized from acetonitrile. The white powder was collected by filtration to give 1.50 g (50%) of celecoxib as a white powder, which was characterized by liquid chromatography (LC) mass spectrometry with electrospray ionization (m/z 380 for $\text{M}+\text{H}^+$) and by ^1H nuclear magnetic resonance (NMR) spectroscopy [(CD_2Cl_2) δ 7.89 (AA'BB', 2 H, $J=2$, 7 Hz), 7.48 (AA'BB', 2 H, $J=2$, 7 Hz), 7.19 (AA'BB', 2 H, $J=2$, 6 Hz), 7.13 (AA'BB', 2 H, $J=2$, 6 Hz), 6.77 (s, 1 H), 4.96 (br s, 2 H), 2.37 (s, 3 H)]. LC mass spectrometry and NMR spectroscopy did not show the presence of any significant detectable impurities (>98% purity). Alternatively, original prescription formulation was also used in the experiments. No difference was detected between effects of purified celecoxib or original formulation with inactive ingredients. All other chemicals were purchased from Sigma Chemical (St. Louis, MO, USA).

Electrophysiology

Whole cell current recordings from retinal neurons and data analysis were performed using an Axopatch 200 amplifier and pClamp 9.2 software (Axon Instruments/Molecular Devices, CA, USA). Patch electrodes were fabricated from thin-walled borosilicate glass (World Precision

Instruments, Sarasota, FL, USA). Electrodes had resistances of 8–10 M Ω . Patch pipettes contained (in mM): 140.0 KCl, 5.4 NaCl, 2.0 MgCl₂, 1.0 CaCl₂, 11.0 EGTA, 10.0 Hepes (pH 7.2). Bath solution contained (in mM): 140.0 NaCl, 4.7 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 10.0 Hepes, and 11.0 glucose (pH=7.4). Experiments were performed either under conditions of continuous exposure of retinal cells to various concentrations of celecoxib in bath solution or using a custom-made perfusion system as indicated in figure legends. The experiments were performed at 21–23 °C. Spontaneous fast current events recorded in the cell-attached mode (“I=0”) were sampled at a frequency of 5 kHz, in the tight seal configuration without rupturing the cell. Third-order retinal neurons with sustained and relatively stable firing rates were used in these experiments.

Microsoft Excel Student’s paired *t*-test was used in the statistical analysis; * *P*<0.05; ** *P*<0.001. Error bars designate S.E.M.

RESULTS

Celecoxib suppresses voltage-gated potassium current in bipolar cells

Retinal bipolar cells are second-order neurons that mediate signal transmission between photoreceptors and the third-order neurons: amacrine and ganglion cells. In the dissociated rat culture, isolated bipolar cells can be identified on the basis of their distinctive morphology and the presence of a characteristic set of voltage dependent currents. These include large, rapidly activating outward potassium currents and small or absent inward sodium currents. Application of celecoxib inhibited the K⁺ currents in all the bipolar cells studied. The inhibition was time-, voltage- and concentration-dependent (Fig. 1B, C, and D respectively). The dose-response relationship of celecoxib’s effect on peak potassium current was fit to the Hill equation yielding an effective concentration to inhibit 50% of function (EC₅₀) of 5.5 μ M (Fig. 1D). Celecoxib reversibly inhibited the K⁺ channels in the bipolar cells from the extracellular side (Fig. 1E). Use of 10 μ M celecoxib in the patch pipette (intracellular) solution did not affect the amplitude or kinetics of the current for as long as the current was monitored, up to 10 min after establishing the whole-cell configuration (intracellular drug duration indicated by dotted line in Fig. 1E). In contrast, the outward current rapidly declined upon the application of the celecoxib to the bath (Fig. 1E, dashed line).

Celecoxib suppresses voltage-gated sodium and potassium currents in third order neurons

Retinal amacrine and ganglion cells are third order retinal neurons. Ganglion cells process visual signals and transmit visual information from the retina to several higher brain centers. Amacrine cells are interneurons, primarily inhibitory. Amacrine and ganglion cells are classified into multiple subtypes with respect to morphology and response to light stimuli. Although ganglion cells in general exhibit larger sodium and potassium currents, it is not possible to unambiguously distinguish between isolated amacrine and ganglion cells on the basis of differences in the ionic currents, and therefore we lumped our study of these cell types into one category: third order neurons. Third order neurons possess prominent voltage-gated sodium currents and a variety of potassium currents, activated directly or indirectly by voltage. Here we describe effects of celecoxib on voltage-gated sodium and potassium currents.

Celecoxib inhibited sodium and potassium currents with time, voltage-, and concentration-dependence. Fig. 2A and B shows recordings from a representative third order neuron in control and in the presence of 10 μ M celecoxib. Both the inward sodium and the outward potassium currents were suppressed by 10 μ M celecoxib. There was a notable time dependent suppression of the potassium current (Fig. 2B). The potassium current in third order neurons consists of a distinct peak transient (I_T) and a sustained K⁺ current (I_S) (Fig. 2A). Fig. 2 illustrates that celecoxib inhibited the sustained outward current (I_S) with a higher potency than the peak

current (I_T). The relative inhibition was voltage dependent for both outward currents (Fig. 2C). The EC_{50} for I_T was slightly higher ($16.3 \pm 2.1 \mu\text{M}$) than that for I_S ($9.1 \pm 1.2 \mu\text{M}$) (Fig. 2D). The time constants for the onset of inhibition (τ_{on}) and the recovery from inhibition (τ_{off}) were 17.0 ± 1.6 and 18.7 ± 5.5 s for I_S , and 26.5 ± 6.2 and 17.8 ± 5.1 s for I_T , respectively. If $10 \mu\text{M}$ celecoxib was added to the pipette's internal solution, the outward potassium current remained unchanged during the course of a recording (Fig. 2F, dotted line). If $10 \mu\text{M}$ celecoxib was then applied to the cell (extracellular application), the potassium current rapidly declined (Fig. 2F, dashed line). The current recovered upon removal of celecoxib from the extracellular bathing solution. The inset shows the potassium currents before (1) extracellular application of celecoxib, during its application (2), and after removal of celecoxib (3). For bipolar cells and third order neurons, celecoxib seemed to act exclusively from the outside membrane surface, indicating that it did not operate by inhibiting cyclooxygenases and suggesting that it might interact directly with sodium and potassium channel proteins.

In whole cell recordings from third order neurons, the sodium current amplitudes varied between 0.3 and 5 nA in different cells and the threshold for activation was between -55 and -40 mV. Celecoxib applied to the extracellular surface of third order neurons reversibly inhibited the Na^+ channels (Fig. 3A). The time constants for the onset of block and the recovery from block were 8.6 ± 1.8 s and 9.7 ± 1.5 s, respectively. Celecoxib significantly decelerated the rate of activation of the Na^+ channels as illustrated in the right panel of Fig. 3A and summarized in Fig. 3B, but did not affect the half-activation potential ($V^{1/2[r]}$). The values of $V^{1/2[r]}$ were -46.5 ± 2.7 mV in control and $-45.232.0$ mV in the presence of $3 \mu\text{M}$ celecoxib. The EC_{50} for the Na^+ conductance suppression was $5.2 \mu\text{M}$ (Fig. 3C). Park et al. (2007) reported that celecoxib suppressed the Na^+ channels in the dissociated rat dorsal root ganglion neurons with a similar potency, although they did not report the slowing of activation kinetics, and the time course of drug action was an order of magnitude faster in our study.

Celecoxib reduces spontaneous activity in third order neurons

Since celecoxib suppressed both voltage-gated sodium and potassium currents, it would likely alter spike activity. But because both currents were suppressed, the effect on spike activity was not straightforward to predict. Whole-cell current clamp mode has been widely used to register action potentials in excitable cells. However, both ruptured- and perforated-patch configurations are associated with changes in the intracellular electrolyte balance and/or loss of cytoplasmic molecules, which can alter the firing properties of neurons (Tabata and Kano, 2002). We used the extracellular cell-attached voltage clamp mode to record capacitive "action currents" produced by action potentials (Feigenspan et al., 1998). This allowed for very stable, non-invasive, and long-lasting recordings. On the other hand, the extracellular recording method does not allow control of membrane potential, triggering of action potentials, or monitoring of non-spiking events in bipolar cells. We therefore evaluated effects of celecoxib in third order neurons with sustained spontaneous firing, and assessed the effects of the drug on the resting membrane potential in a separate set of experiments in the ruptured-patch configuration. In general large neurons were selected and approximately 30% of these cells displayed irregular spontaneous firing between 0.25 and 12 Hz, with an average rate of 1.9 ± 0.2 Hz, similar to previous observations (Feigenspan et al., 1998). Fig. 4A shows a representative example of the spontaneous spike pattern in control, after 1 min in the presence of $3 \mu\text{M}$ celecoxib, and 1 min after removal of celecoxib. Celecoxib reduced the rate of spontaneous activity in a concentration-dependent manner with an EC_{50} of $0.76 \mu\text{M}$ and almost complete block at $10 \mu\text{M}$ celecoxib (Fig. 4B and C). Since the firing was irregular, it was particularly important to demonstrate that the inhibition was reversible. Fig. 4B and 5A show that the effects were reversible at all concentrations tested. In a separate set of experiments, the resting membrane potentials (RMP) of third order neurons were monitored using whole-cell current clamp ($I=0$). The mean resting membrane potential was -53.5 ± 3.8 mV, typical for this age and

preparation (Reiff and Guenther, 1999), and was not significantly altered by celecoxib (Fig. 4D).

Fig. 5A shows that the onset of spike suppression occurred within seconds after the start of celecoxib application which is consistent with the time constants for inhibition of sodium and potassium currents (Figs. 2 and 3). Given that celecoxib can inhibit sodium and potassium currents, it was informative to compare the effect of specific channel blockers on the sustained spike activity as measured using action currents. Not unexpectedly, spike activity was fully suppressed by 300 nM tetrodotoxin (TTX) (Fig. 5B). The firing was slowly re-established after wash-out. Non-specific block of voltage-gated calcium and calcium-activated potassium channels with cadmium did not diminish the spike frequency (Fig. 5C). Tetraethylammonium (TEA) blocks voltage-gated and calcium-activated potassium channels, but did not reduce spike activity in third order neurons as shown in Fig. 5D. The fast transient potassium current blocker 4-aminopyridine (4-AP) did not decrease spike activity (Fig. 5E). Thus, the spike activity suppression was not due to a depolarizing block resulting from the inhibition of potassium channels. TEA and 4-AP, but not celecoxib, altered the shape of the action currents. 4-AP completely abolished, while TEA distorted and significantly prolonged the after-hyperpolarization phase of action potential (Fig. 5G and H). The time course of these events was fast, while the normal interspike interval was long. This is probably the reason that potassium channel block had little effect on spontaneous spike frequency.

DISCUSSION

These experiments demonstrate that celecoxib suppresses voltage-gated sodium current and two types of potassium current in retinal neurons. Several other recent studies have also demonstrated that celecoxib at low micromolar concentrations can inhibit ion channels including voltage-activated sodium, calcium, and potassium channels (Frolov et al., 2008; Park et al., 2007; Zhang et al., 2007). In *Drosophila* muscle, celecoxib suppressed various channels with different potencies. Celecoxib inhibited the I_{KS} (quinidine-sensitive, Kv2) potassium current with an EC_{50} of 11.4 μ M (Frolov et al., 2008). It also inhibited an L-type calcium current with an IC_{50} of 74 μ M, and I_A (4-AP-sensitive Kv1) potassium current with an EC_{50} near 100 μ M (R. V. Frolov and S. Singh, unpublished observations). In retina, the range of potencies was much narrower, ranging from 5 to 16 μ M celecoxib.

In isolated rat cardiac muscle fibers, the rate of spontaneous contraction was reduced by celecoxib (Frolov et al., 2008). Similarly, in rat retinal third order neurons, celecoxib inhibited spontaneous spike firing. Inhibition of the sustained spike activity in third-order neurons was due primarily to blockade of sodium channels as evidenced by experiments with TTX and the ineffectiveness of potassium channel blockers. Blockade of the voltage-activated potassium channels by TEA is known to reduce the rate of induced action potential firing in neurons with high spike frequencies, between 40–150 Hz (Kasten et al., 2007). Although our data confirm that TEA and 4-AP can alter the shape of action potential waveform, they probably did not affect spike frequency because of the very low rate of spontaneous activity in the present study (1.9 ± 0.2 Hz). Similarly, use of 4-AP, TEA, and calcium channel blockers did not inhibit spontaneous firing in the isolated mouse retinal third-order neurons (Feigenspan et al., 1998).

Importantly, we found a sevenfold difference between half-maximal concentrations for inhibition of sodium channels (5.2 μ M celecoxib) and spontaneous firing in the third order retinal neurons (0.76 μ M celecoxib). A similar sevenfold relationship between blockade of the sodium channels by lidocaine (210 μ M) and inhibition of the neuronal function mediated by these channels (30 μ M lidocaine) has been reported (Scholz and Vogel, 2000). These observations may signify that a small change in the number of functional channels (9% of the sodium channels are blocked by 0.76 μ M celecoxib based on data from Fig. 3C) can profoundly

affect the level of neuronal activity. It is likely that both reduction in the number of sodium channels and slowing of their activation kinetics disrupt the regenerative initiation of action potentials. Since spike frequency modulation is the paramount means of information processing in the CNS, celecoxib may modulate neural networks. Whether this contributes to the analgesic action of celecoxib is unknown.

The effects of celecoxib on spontaneous sustained activity in the third order retinal neurons may be clinically relevant. A 25% reduction in the level of sustained firing was detected at 300 nM celecoxib. Various studies show that the peak celecoxib concentration in human plasma is between 1.7 and 2.8 μM after the minimal adult therapeutic dose of 200 mg (Davies et al., 2000). The drug is generally prescribed at 200 mg twice daily (Solomon et al., 2005). Doses of 400 mg or 600 mg twice daily have also been administered to patients or used in clinical trials (Solomon et al., 2005). Plasma concentration is higher in many conditions. For example, a single dose of 200 mg gives an average concentration of 6.2 μM in women aged 65 or above (Davies et al., 2000). In addition, moderate hepatic impairment or co-administration of certain drugs, such as ketoconazole, increases the plasma concentration severalfold (Davies et al., 2000). Two factors, however, complicate estimation of the actual concentration of celecoxib in blood and tissues. On one hand, celecoxib binds to plasma proteins, with the free plasma concentration equal to 3% of the total plasma concentration (Davies et al., 2000). On the other hand, the average volume of distribution for celecoxib is 455 L which means an extensive tissue deposition of the drug (Davies et al., 2000). The situation is further complicated by individual to individual variations in drug-absorption and drug-response. With tens of millions of people using the drug, the potential exists for clinically prescribed doses of celecoxib to affect neuronal activity and to lead to heretofore-unanticipated effects. A few case studies indicate that coxibs may have adverse effects on vision (Coulter et al., 2003; Fraunfelder et al., 2006). Whether these cases reflect factors discovered in these experiments is unknown.

Acknowledgements

We are particularly thankful to Dr. Michael R. Detty, Mr. Bryan R. Wetzel and Mr. Michael K. Gannon for purification and characterization of celecoxib. We are thankful to Mr. Jaeyoung Yang for the isolation of rat retinal cells, and Mr. Anil Neelakantan for helpful discussions. This work was supported by NEI grant 05725 to M.M.S. and NSF grants MCB-0094477 and MCB-0322461 to S.S.

Abbreviations

EC₅₀	effective concentration to inhibit 50% of function
EDTA	ethylenediaminetetraacetic acid
I_S	sustained current
I_T	fast transient current
LC	liquid chromatography
NMR	nuclear magnetic resonance
TEA	tetraethylammonium

TTX

tetrodotoxin

V^a_{1/2}

half-activation potential

4-AP

4-aminopyridine

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Fig. 1.

Effects of celecoxib on potassium currents in bipolar cells. Outward potassium currents in control (A) and in the presence of 3 μM celecoxib (B) elicited by 100-ms depolarizing pulses from a holding potential (HP) of -70 mV to +60 mV applied in 10 mV increments. (C) Normalized average current-voltage (I-V) relations are shown in control and in the presence of 3 μM celecoxib. (D) The celecoxib dose-response relationship for the peak potassium currents at +40 mV. The data were fit to the equation $f(D)=1/[1+(D/\text{EC}_{50})^n]$, where D is the concentration, n is the Hill coefficient, and EC_{50} is the concentration of half-suppression; number of cells for each data point varied from four to eight. (E) A representative time course of the K^+ current inhibition obtained using a 0.2 Hz train of 100-ms pulses (-70 mV to +40 mV). The presence of 10 μM celecoxib in the patch pipette solution (dotted line) did not affect the K^+ current, but the external application of 10 μM celecoxib (dashed line) rapidly and reversibly inhibited the current. Inset: the current responses for the designated time points during experiment.

Fig. 2.

Effects of celecoxib on the potassium currents in the third order retinal cells. The current in control (A) and at 10 μ M celecoxib (B) was evoked by 100-ms voltage pulses between -60 and +60 mV (HP=-70 mV) in 10 mV increments. (C) Normalized averaged current-voltage (I - V) relations are shown for the peak K^+ current (I_T) and the current at the end of the 100-ms pulse (I_S) in control and in the presence of 10 μ M celecoxib. (D) The dose-response relationships for I_T and I_S at +40 mV (HP=-70 mV); number of cells per data point varied from 8 to 12. (E) The I_T was less sensitive to inhibition by celecoxib (dashed line) than the I_S at 10 μ M celecoxib (legends as in D). (F) The presence of 10 μ M celecoxib in patch pipette solution (dotted line) did not affect the K^+ current, but the external application of 10 μ M celecoxib (dashed line) rapidly and reversibly inhibited the current. Inset: the current responses for the designated time points during experiment.

Fig. 3.

Effects of celecoxib on the sodium currents in the third order retinal cells. (A) The extracellular (dashed line) application of 10 μ M celecoxib inhibited the sodium current reversibly; the presence of 10 μ M celecoxib in the patch pipette solution (dotted line) did not affect the current. The sodium current was elicited using a 0.5 Hz train of 20-ms depolarizing pulses from a HP of -70 mV to -40 mV. The current traces are shown for the designated time points during experiment. (B) Celecoxib reversibly decelerated the activation kinetics of the sodium channels. The τ_{act} values were determined by fitting the rising phase of the sodium current evoked at -40 mV (HP= -70 mV) with a function $f_a(t)=C(1-\exp(-t/\tau_{act}))^3$. (C) The dose-response relationship for celecoxib's effect on the maximal sodium conductance; number of cells per data point varied from 5 to 12.

Fig. 4.

Effects of celecoxib on spontaneous action potentials in the third order retinal neurons. (A) An example of temporal patterns of spontaneous activity in control, in the presence of 3 μ M celecoxib, and after washout. (B) Bar plot shows the dose-dependence and reversibility of celecoxib's effect, number of cells per data point varied from 6 to 14. (C) The concentration dependence of the spontaneous firing rate suppression by celecoxib was fit to the Hill equation. (D) Celecoxib at 3 μ M did not affect the resting membrane potential in the third order neurons. The data were obtained from a separate pool of cells during whole-cell voltage clamp experiments ($n=6$).

Fig. 5.

Temporal patterns of the spontaneous firing in the presence of 10 μ M celecoxib (A), 300 nM TTX (B), 500 μ M CdCl₂ (C), 20 mM TEA (D), and 2 mM 4-AP (E). The effects of celecoxib (F), TEA (G), and 4-AP (H) on the shape of the averaged action currents observed in the cell-attached configuration with the pipette potential held at 0 mV before drug application (1), in the presence of a drug (2), and after washout (3).