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Positive Regulation by Chloride Channel Blockers of I_{sK} Channels Expressed in *Xenopus* Oocytes

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SUMMARY

cRNA encoding the human I_{sK} protein was injected into *Xenopus* oocytes and the induced I_{sK} channels were investigated using the two-microelectrode voltage-clamp method. Niflumic acid, mefenamic acid, flufenamic acid, and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid, which are commonly used in *Xenopus* oocytes to suppress endogenous Ca^{2+}-activated Cl^- channels, were tested for their effects on I_{sK} channels. At low concentrations (10 μM) all compounds increased I_{sK} amplitude and decreased the rate of I_{sK} deactivation. At 100 μM these compounds further decreased the rate of I_{sK} deactivation, resulting in persistent activation of I_{sK}, similar to what has been previously described for the action of organic cross-linkers on I_{sK}. However, at 100 μM niflumic acid and flufenamic acid decreased the time-dependent outward current, whereas 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid and mefenamic acid caused an additional increase. When Cl^- was completely substituted with gluconate, I_{sK} had somewhat altered activation properties, but niflumic acid produced similar positive regulatory effects on I_{sK} and shifted the voltage needed to evoke half-maximal I_{sK} activation ($V_{½}$) by about −20 mV. In summary, these compounds positively regulate I_{sK}, presumably by stabilizing open I_{sK} channels.

Expression of the I_{sK} protein in *Xenopus* oocytes induces a slowly activating, voltage-dependent, K^+ current, I_{sK} (1). In addition to the slow kinetics of I_{sK}, the protein inducing the current has a unique molecular structure with only one putative membrane-spanning domain. Although it has recently been suggested that the I_{sK} protein may act as an ion channel regulator (2), there is overwhelming evidence for the I_{sK} protein being an integral part of an ion channel itself (3–6). I_{sK} channels expressed in *Xenopus* oocytes have a characteristic pharmacological profile. They are moderately sensitive or are insensitive to the classical K^+ channel blockers Ba^{2+}, tetraethylammonium, 4-aminopyridine, and clofilium (1, 7) and are highly sensitive to the novel class III antiarrhythmic agent NE-10064 (8) and calmodulin antagonists (9). Because in *Xenopus* oocytes Ca^{2+}-activated Cl^- channels can interfere with the activation of expressed ion channels and make an analysis of their activation kinetics difficult, Cl^- channel blockers such as fenamates (10) are widely used to filter out Cl^- currents. However, their use could be restricted if these compounds alter other ion conductances. Indeed, fenamates are known to also inhibit cation channels (11), and preliminary data indicated an action on Ca^{2+}-activated K^+ channels (12). The aim of this study was to test for putative effects of such blockers on I_{sK} channels expressed in *Xenopus* oocytes.

Experimental Procedures

Handling and injection of *Xenopus* oocytes have been previously described in detail (13). The two-microelectrode voltage-clamp configuration was used to record currents from *Xenopus* oocytes that had been previously injected with cRNA transcribed *in vitro* from a cDNA isolated from human genomic DNA (14). In a different set of experiments the rat I_{sK} protein was also expressed (13). Recordings were performed at room temperature. If not otherwise stated, I_{sK} was activated with 15-sec voltage steps to −10 mV. The holding potential was −80 mV for all experiments. To test for instantaneous (persistently activated) currents, a voltage-step family with 200-msec voltage steps every 1 sec from −110 mV to −60 mV, with increments of 10 mV, was performed 15 sec after such depolarizations. The superfusing solution contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, titrated to pH 7.4 with NaOH. In some experiments Cl^- was substituted in the bath solution with the corresponding gluconate salts and in the recording pipette with 1 M potassium gluconate. I_{sK} current amplitude and the activation and deactivation rates depend on the time of I_{sK} protein expression (15). Experimental and control data were therefore always obtained in oocytes from one frog on one specific day. All experiments were repeated on different days and always gave qualitatively similar results. Chemicals were added from stock solutions into the superfusion solution as indicated. Chemicals used were niflumic acid, mefenamic acid, flufenamic acid, DIDS, acetylsalicylic

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ABBREVIATIONS: I_{sK}, slowly activating, voltage-dependent K^+ current; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

acid, acetophenitidin, and A23187 (Sigma). Activation and deactivation time constants were obtained by fitting a two-exponential function to the outward traces (15) and a single-exponential function to the tail currents, respectively. Data are presented as means ± standard errors, where n denotes the number of experiments performed. The paired t test was performed to test for statistical significance. Data were considered significantly different at $p < 0.05$.

Results and Discussion

cRNA encoding the human I_{sK} protein was injected into *Xenopus* oocytes and the induced I_{sK} channels were investigated using the two-microelectrode voltage-clamp method. I_{sK} was evoked with 15-sec voltage steps to −10 mV, conditions under which no significant endogenous Cl^- current was observed. Addition of niflumic acid (10 μM) to the bath solution increased I_{sK} by about 16% and decreased the rate of I_{sK} deactivation at −80 mV (τ_{deact}) by 58% (Fig. 1, A and B; Table 1A). The fenamates mefenamic acid and flufenamic acid and the chemically unrelated Cl^- channel blocker DIDS had qualitatively similar effects on I_{sK}; they increased I_{sK} and decreased its rate of deactivation (Table 1). Recently, for rat I_{sK} channels a higher sensitivity to La^{3+} blockade and a slower deactivation rate, compared with human I_{sK} channels, have been described (6). In this study, however, superfusion of oocytes expressing the rat I_{sK} protein with 10 μM niflumic acid resulted in an increase of the outward current and a decrease of the rate of rat I_{sK} deactivation. Rat I_{sK} was increased by 30% (from 245 ± 71 nA under control conditions to 320 ± 93 nA after niflumic acid, n = 4) and τ_{deact} was increased by 35% (from 1105 ± 192 msec to 1492 ± 256 msec, n = 4), in spite of the difference in the deactivation rates for rat and human I_{sK} under control conditions.

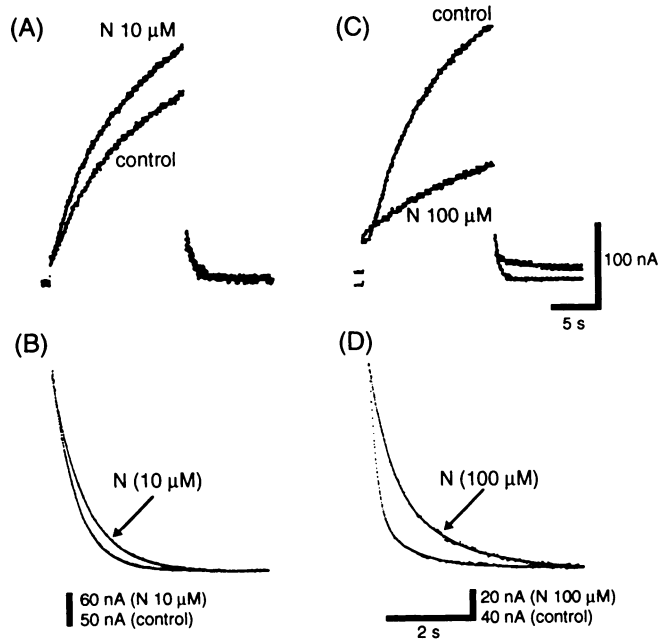

Fig. 1. Effects of niflumic acid (*N*) at 10 and 100 μM on I_{sK} amplitude and tail currents (I_{sK} was activated by 15-sec voltage steps from −80 to −10 mV). A and B, Niflumic acid (10 μM) increases I_{sK} (A) and at repolarization to −80 mV decreases its rate of deactivation (B). C and D, Niflumic acid (100 μM) decreases I_{sK}, shifts the holding current in the outward direction (C), and decreases further I_{sK} deactivation (D). Please note that the superimposed tail currents were scaled to give the same tail amplitude. B and D show single-exponential fits to the current traces.

TABLE 1

Effects of Cl^- channel blockers on amplitude and deactivation rate of human I_{sK}

Human I_{sK} was measured after 15-sec depolarizing steps to −10 mV. I_{sK} deactivation was recorded at the holding potential of −80 mV. The current amplitudes and deactivation time constants were individually normalized against the control amplitudes. The data give the arithmetic means ± standard errors.

Cl^- channel blocker	$I/I_{control}$	$\tau_{deact}/\tau_{deact,control}$	n
A. 10 μM			
Niflumic acid	1.16 ± 0.03*	1.58 ± 0.19*	7
Flufenamic acid	1.20 ± 0.03*	1.23 ± 0.11*	6
Mefenamic acid	1.19 ± 0.03*	1.85 ± 0.13*	8
DIDS	1.41 ± 0.15*	2.14 ± 0.38*	5
B. 100 μM			
Niflumic acid	0.55 ± 0.06*	2.94 ± 0.11*	6
Flufenamic acid	0.26 ± 0.00*		4
Mefenamic acid	2.39 ± 0.01*	17.8 ± 1.3*	4
DIDS	2.95 ± 0.03*	12.0 ± 0.9*	
C. 10 μM in Cl^--free solution			
Niflumic acid	1.56 ± 0.05*	1.64 ± 0.23*	4

* Statistically significant difference ($p < 0.05$) from control.

At 100 μM niflumic acid decreased the rate of deactivation even more strongly (Fig. 1D; Table 1B). However, niflumic acid (Fig. 1C) and flufenamic acid decreased the time-dependent outward K^+ current at this concentration by 45 and 74%, respectively (n = 6 and 4, respectively). This inhibitory effect indicates an additional compound-channel protein interaction, causing channel blockade. The nature of this blockade remains unclear, however. In contrast, as shown in Fig. 2, the positive regulatory effects of mefenamic acid and DIDS were enhanced at 100 μM. The time-dependent current was increased 2–3-fold (Fig. 2A; Table 1B) and I_{sK} deactivation could no longer be described with a single-exponential function but was composed of two distinct, very slow events. The τ_{deact1} value, reflecting the faster deactivation rate, was 10–20-fold larger with 100 μM DIDS and mefenamic acid. The τ_{deact} value was increased from 0.24 ± 0.04 sec and 0.28 ± 0.01 sec in controls to 3.00 ± 0.04 sec and 4.98 ± 1.31 sec after DIDS and mefenamic acid, respectively (n = 4 for each group). The second event was even slower, with an estimated τ_{deact2} of >30 sec, resulting in persistently opened I_{sK} channels during repetitive depolarizations. As a result, the holding current at −80 mV shifted in the outward direction (Fig. 2A). By performing short voltage steps (see Experimental Procedures), no significant current could be recorded under control conditions. However, using the same protocol with 100 μM DIDS an instantaneous current was observed, which reversed at −97 mV, indicating its selectivity for K^+ ions (Fig. 2, B and C). The DIDS-mediated instantaneous K^+ current was greatly reduced after addition of the I_{sK} channel blocker NE-10064 (10 μM) (Fig. 2, B and C) (8). Qualitatively similar results were obtained with mefenamic acid (100 μM; data not shown). These data strongly suggest that DIDS and mefenamic acid can indeed stabilize open I_{sK} channels, causing a persistent activation of I_{sK}. Interestingly, at high concentrations there was a qualitative difference between the effects of fenamates with a -CF_3 group on the phenyl ring (niflumic acid and flufenamic acid) and those of mefenamic acid, which has two methyl groups on the phenyl ring. Whereas niflumic acid and flufenamic acid at high concentrations caused an additional inhibitory effect on I_{sK}, no inhibition but an increase of the positive regulatory effect was observed with mefenamic acid.

To avoid Cl^- conductance interference and to study I_{sK}

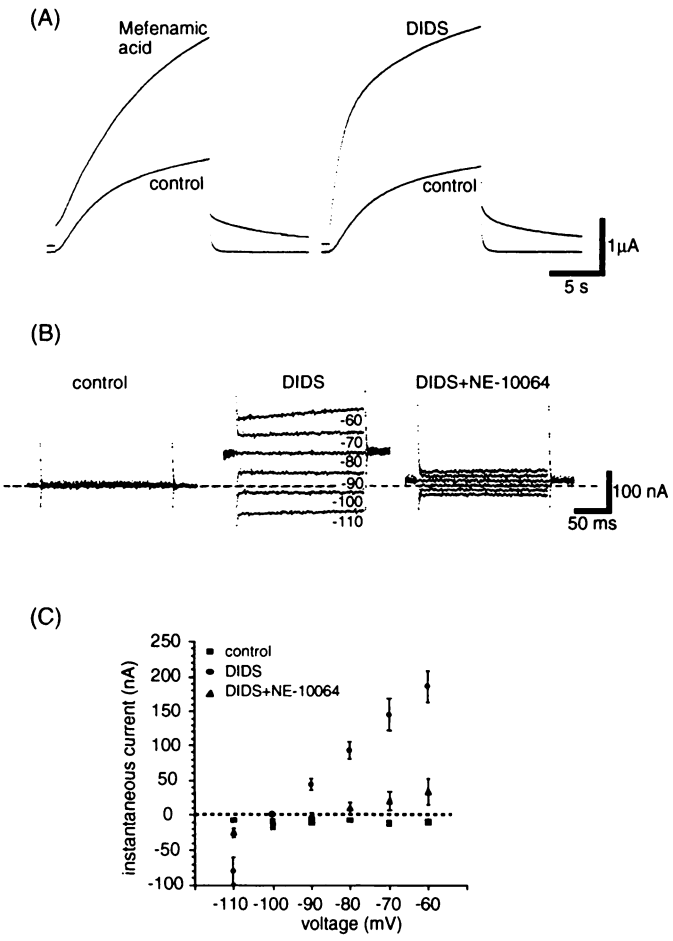

Fig. 2. Effects of mefenamic acid and DIDS (each at 100 μM) on I_{sK}. A, Both mefenamic acid (*left*) and DIDS (*right*) increase I_{sK}, shift the holding current in the outward direction, and decrease the rate of I_{sK} deactivation. B, A voltage step family (as indicated in *middle*) was performed 15 sec after a depolarizing voltage step (15 sec, to −10 mV) (see Experimental Procedures). Under control conditions (*left*) no significant current was recorded. However, after DIDS an instantaneous current was observed (*middle*), which reversed at about −97 mV and was greatly inhibited by 10 μM NE-10064 (*right*). *Dashed line*, 0 current. The holding potential was −80 mV. C, I–V relationship of (B).

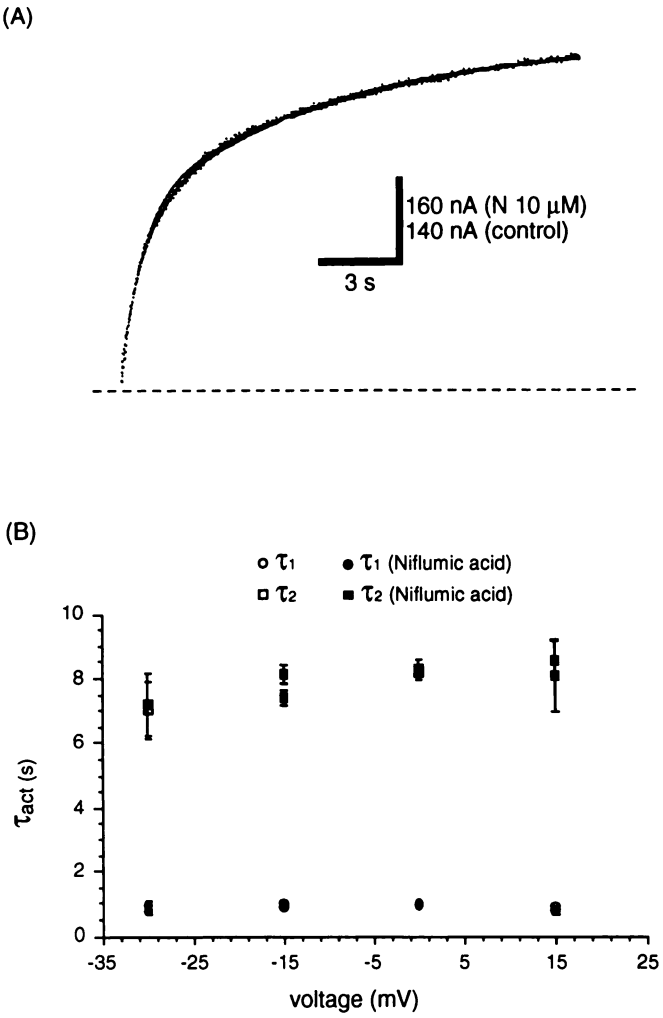

Fig. 3. Lack of effect of niflumic acid on I_{sK} activation under Cl^--free conditions. A, Superimposed traces of I_{sK} evoked by a 20-sec depolarizing step from −80 to 0 mV under control conditions and with 10 μM niflumic acid. The traces were scaled to give the same amplitude and were fitted by a two-exponential function. B, I_{sK} activation time constants plotted against the voltage.

activation in more detail, one set of experiments was performed under Cl^--free conditions (microelectrodes were filled with 1 M potassium gluconate solution, in the bath solution all Cl^- was substituted with gluconate, and oocytes were stored in a Cl^--free solution for 1 hr before the experiments). Under these conditions no Ca^{2+}-activated Cl^- current could be recorded by performing voltage steps to voltages from −20 to 40 mV (n = 6) or by superfusion with the Ca^{2+} ionophore A23187 at a concentration of 1 μM (n = 6; data not shown). I_{sK} was activated somewhat faster in Cl^--free solution than under control conditions. Fitting a two-exponential function to the currents resulted in a fast time constant (τ_1) of about 0.9 sec and a slow time constant (τ_2) of about 8 sec, contributing 41 and 59%, respectively, of the outward current at 0 mV. Because the depolarizing steps lasted only 20 sec, τ_2 can be taken only as a rough estimate. At voltages from −35 to 15 mV, niflumic acid (10 μM) did not affect the rate of I_{sK} activation (Fig. 3). Long depolarizing steps to voltages higher than 15 mV could not be performed because under such conditions an endogenous, slowly activating Na^+ channel was evoked (16). However, under Cl^--free conditions niflumic acid (10 μM) still increased I_{sK} and τ_{deact}. The effects of niflumic acid were enhanced at more negative potentials and were not significant when I_{sK} was evoked by depolarizing steps to voltages of ≥15 mV (Fig. 4A). This was reflected in the current-voltage relationship, where the estimated voltage needed to evoke half-maximal I_{sK} activation ($V_{1/4}$) was shifted by about −20 mV after addition of niflumic acid (10 μM) to the bath solution (Fig. 4B).

Fenamates are known to inhibit cyclooxygenase. However, acetylsalicylic acid and acetophenitidin (phenacetin), two other cyclooxygenase inhibitors, did not affect I_{sK} at concentrations up to 100 μM (data not shown). Inhibition of cyclooxygenase is therefore not likely to be the mechanism for the observed I_{sK} regulation.

In water-injected oocytes as well as in oocytes expressing the I_{sK} protein, voltage steps to potentials greater than −20 mV resulted in Ca^{2+}-activated outward Cl^- currents. This outwardly directed Cl^- current was completely suppressed in the presence of niflumic acid (100 μM; n = 6), similar to what was previously reported by White and Aylwin (10). Because the Cl^- currents

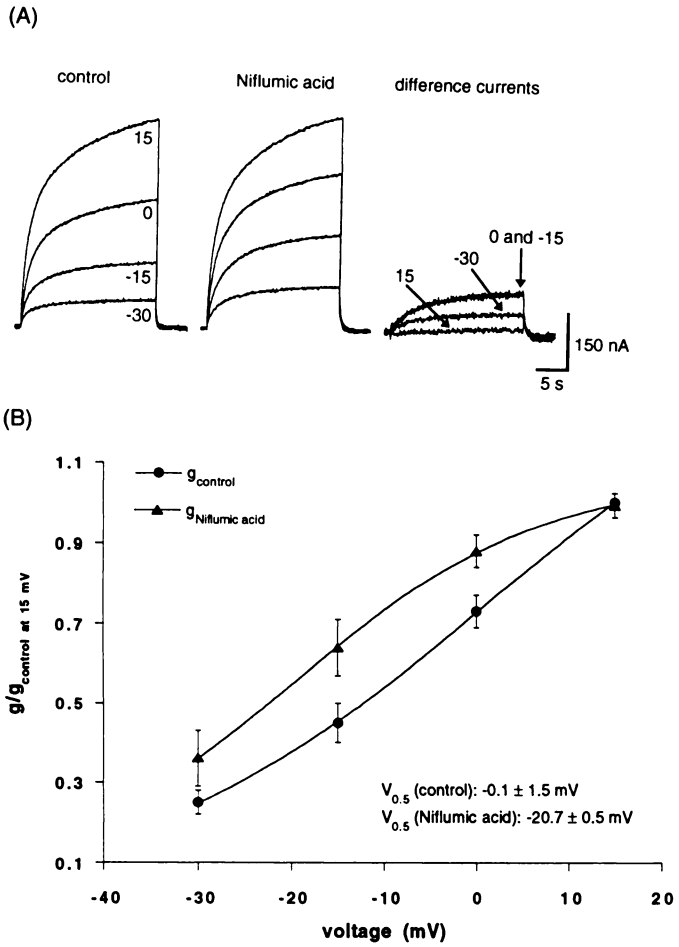

Fig. 4. Effects of niflumic acid on I_{sK} at different potentials in a representative oocyte under Cl^--free conditions. A, I_{sK} evoked with 20-sec voltage steps from −80 mV to the potentials indicated. The "difference currents" were obtained by subtracting the control currents from the niflumic acid-regulated currents. B, Normalized conductance-voltage relationship of I_{sK} before and after superfusion with 10 μM niflumic acid under Cl^--free conditions. The conductance was calculated assuming an E_K of −100 mV.

were outwardly directed at −10 mV and were inactivated almost completely during the usual 15-sec voltage steps, inhibition of these endogenous currents can be ruled out as an explanation for the I_{sK} increase. Furthermore, none of the compounds activated any other ion conductances or increased an outward current in water-injected oocytes ($n = 7$; data not shown).

I_{sK} has been shown to be positively regulated by oocyte swelling (17). Inhibition of endogenous Cl^- channels could result in oocyte swelling and subsequently lead to positive I_{sK} regulation. Three arguments oppose this hypothetical mechanism. (a) The effects of the Cl^- channel blockers were exerted at concentrations below their EC_{50} values for Cl^- channels (10). (b) Niflumic acid (10 μM) positively regulated I_{sK} under Cl^--free conditions. (c) Swelling of oocytes results in accelerated I_{sK} activation without changes in τ_{deact} (17), which is in contrast to the effects of niflumic acid on I_{sK}, where activation was unchanged whereas τ_{deact} was increased.

The effects occurred rapidly, which further indicates direct binding to the channel protein rather than an indirect regulatory effect. The regulation of I_{sK} produced by these compounds was qualitatively similar to what has been previously described for membrane-impermeable organic cross-linkers (18). It is therefore possible that fenamates and DIDS act by forming complexes with I_{sK} proteins, causing stabilization of open I_{sK} channels. Fenamates are still therapeutically used as analgesic and anti-inflammatory compounds, and a possible activation of K^+ channels by these Cl^- channel inhibitors could be of pharmacological interest and may be responsible for some of the side effects observed with these compounds (19).

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