Effect of isosorbiddinitrate on exogenously expressed slowly activating K^+ channels and endogenous K^+ channels in *Xenopus* oocytes

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1. The effects of isosorbiddinitrate (ISDN) were tested on membrane currents and resting potential in *Xenopus laevis* oocytes which were either uninjected or injected with cRNA encoding for K^+ channels from three distinct families (slowly activating I_{sK} channels, delayed-rectifying Kv1.1 or inwardly rectifying IRK1 K^+ channels).

2. In uninjected oocytes ISDN (1 mM) resulted in a decrease of the holding current at potentials more positive than −100 mV and in an increase at potentials below −100 mV. Increasing extracellular K^+ to 100 mM shifted the reversal potential for ISDN-mediated effects to approximately −12 mV, suggesting an inhibition of a K^+ conductance by ISDN.

3. In current clamp studies ISDN (1 mM) and Ba^{2+} (3 mM) depolarized cell membrane. ISDN and Ba^{2+} had no additive effects on membrane potential when applied simultaneously. In voltage clamp studies, corresponding results were observed for the effects of ISDN and Ba^{2+} on the holding current with an apparent K_m of 0·21 and 0·08 mM, respectively.

4. In contrast to ISDN, the nitric oxide (NO) donors isosorbidmononitrate (ISMN) and *S*-nitrosocysteine (SNOC) had no effects on the holding currents in *Xenopus* oocytes. Moreover, the guanylate inhibitor LY 83583 did not affect ISDN-mediated holding current alterations, suggesting that ISDN acts independently of the second messenger NO.

5. ISDN inhibited exogenously expressed I_{sK} channels with an apparent K_m of 0·15 mM, but at 1 mM only weakly inhibited Kv1.1 and IRK1 channels.

6. It is concluded that ISDN inhibits an endogenous K^+ conductance in *Xenopus* oocytes with a similar potency to that shown by expressed I_{sK} channels. These effects are independent of the second messenger NO.

Nitric oxide (NO) is an important second messenger in a variety of tissues (see Moncada, Palmer & Higgs, 1991) and has been shown to exert its effects via activation of guanylate cyclase (see Furchgott, 1990) and/or direct oxidation of target proteins such as the NMDA receptor (Lei, Pan, Aggarwal, Chen, Hartman, Sucher & Lipton, 1992) and the calcium-activated K^+ channel of vascular smooth muscle (Bolotino, Njibi, Palaino, Pagano & Cohen, 1994). In smooth muscle NO is the endothelium-derived relaxing factor (EDRF) (see Moncada *et al.* 1991). NO is physiologically synthesized from L-arginine (see Moncada, Palmer & Higgs, 1989) requiring tissue thiols to form enzymatically *S*-nitrosothiols as intermediates (Ignarro *et al.* 1981). The cellular synthesis of NO from pharmacologically useful organic nitrates, such as isosorbiddinitrate (ISDN) or nitroglycerin, is therapeutically important in the treatment of coronary heart disease. Synthesis of NO from organic nitrates requires a sophisticated enzyme system (Feelisch & Noack, 1991) similar to the physiological synthesis of NO from L-arginine (Ignarro *et al.* 1981). Effects of organic nitrates independent of the second messenger NO have not as yet been described. The aim of these experiments was to determine the effects of the organic NO donors ISDN, isosorbidmononitrate (ISMN) and *S*-nitrocysteine (SNOC) on *Xenopus* oocytes, cells widely used for the expression and characterization of exogenous proteins (Dascal, 1987). Furthermore, we were interested in the effects of organic nitrates on distinct classes of K^+ channels expressed after cRNA injection.

METHODS

Handling and injection of *Xenopus* oocytes and synthesis of cRNA has been described previously in detail (Christie, Adelman, Douglass & North, 1989; Busch, Kavanaugh, Varnum, Adelman & North, 1992). *Xenopus laevis* were anaesthetized in a 3-amino-benzoic acid ethyl ester solution (1 g l^{-1}) and put on ice. A small incision was made to retrieve sacs of oocytes. The incision was sutured with absorbable surgical suture and the *Xenopus* was put back in the aquarium for at least 3 weeks. In general we retrieved three batches of oocytes from one *Xenopus*. After the third dissection the frog was killed under anaesthesia by decapitation. The sacs of oocytes were torn and the oocytes were first washed five times in a Ca^{2+}-free ND96 solution and subsequently collagenized in a ND96-containing collagenase A (Boehringer Mannheim; 1 g l^{-1}) until no follicle was detectable on the surface of the oocytes. After defolliculation the oocytes were washed thoroughly with ND96 solution and kept at 16 °C. One day after dissection oocytes were selected and for certain experiments injected with the appropriate cRNA. The two-microelectrode voltage or current clamp configuration was used to record currents or cell membrane potential changes from *Xenopus laevis* oocytes. In several sets of experiments oocytes were individually injected with cRNA encoding for the K^+ channels human I_{sK} (Murai, Kakizuka, Takumi, Ohkubo & Nakanishi, 1989), rat Kv1.1 (Christie *et al.* 1989) or rat IRK1 (Fakler, Brändle, Glowatzki, Zenner & Ruppersberg, 1994). Recordings were performed at 22 °C using a Geneclamp amplifier (Axon Instruments) and MacLab D/A converter and software for data acquisition and analysis (AD Instruments, Castle Hill, NSW, Australia). Outward currents through I_{sK} or Kv1.1 channels were evoked with 15 or 0·5 s depolarizing pulses to −10 mV from a holding potential of −80 mV, and filtered at 10 Hz and 1 kHz, respectively. K^+ inward currents through inwardly rectifying IRK1 channels were evoked by hyperpolarizing the cells for 0·5 s to −120 mV from a holding potential of −40 mV (filtered at 1 kHz). The amplitudes of the recorded currents were measured at the end of the test voltage steps. The control solution (ND96) contained (mM): NaCl, 96; KCl, 2; $CaCl_2$, 1·8; $MgCl_2$, 1; Hepes, 5 (titrated with NaOH to pH 7·5). As indicated below, in some experiments Na^+ was replaced by K^+ (100 mM K^+ solution), Na^+ was reduced to 10 mM by substitution with choline (10 mM Na^+ solution), Cl^- was replaced by gluconate (0 mM Cl^- solution), or the solution contained no Ca^{2+} (0 mM Ca^{2+} solution). The microelectrodes were filled with 3 M KCl solution and had resistances between 0·5 and 0·9 MΩ. Chemicals used

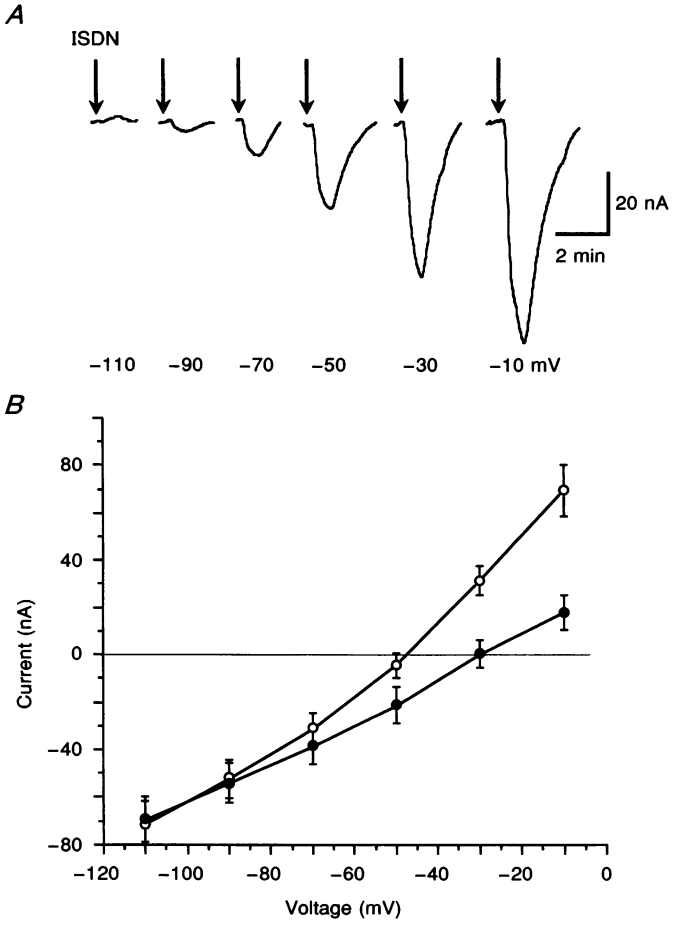

Figure 1. Effects of ISDN on oocyte membrane currents

A, *Xenopus* oocytes were voltage clamped at the indicated potentials. The arrows indicate the start of a 30 s superfusion period with ISDN (1 mM). The ISDN-mediated effects reverse between −110 and −90 mV. To clarify the effects of ISDN, the holding currents at the different potentials were aligned. The approximate values for the holding currents can be taken from *B*. *B*, the arithmetic means (± S.E.M.) of the steady-state holding currents between −110 and −10 mV (○) and the alterations caused by ISDN (●).

were: ISDN, L-cystein and $NaNO_2$ (for the synthesis of *S*-nitrosocystein, see Raber *et al.* 1995) and LY 83583 from Sigma; ISMN was a generous gift from Mack, Illertissen, Germany. Data are presented as means ± standard error of the mean (S.E.M.), where n represents the number of experiments performed. Student's paired t test was used to test for statistical significance, which was obtained for $P < 0{\cdot}05$.

RESULTS

Effects of ISDN on endogenous currents in *Xenopus* oocytes

Steady-state holding currents in *Xenopus* oocytes ranged in control solution from −70 to + 70 nA at −110 and −10 mV holding potential, respectively, reversing their direction at approximately −48 mV (Fig. 1*B*; $n = 8$). At potentials between −90 and −10 mV superfusion with ISDN (1 mM) shifted the holding current to more negative values. However, at potentials more negative than −100 mV, ISDN shifted the holding current in an outward direction (Fig. 1*A*). Under ISDN the reversal potential of the holding currents was approximately −32 mV (Fig. 1*B*; $n = 8$). ISDN effects occurred rapidly and were promptly and completely reversible upon washout.

Changes in holding current (ΔI) due to ISDN were studied under distinct ionic conditions. Increasing extracellular K^+ to 100 mM (by replacing NaCl with KCl) caused a shift of the reversal potential for ISDN-mediated ΔI from −100 to approximately −12 mV (Fig. 2*A* and *B*; $n = 5$). In contrast, reducing Na^+ to 10 mM or withdrawal of Ca^{2+} and Cl^- from the extracellular solution did not affect the amplitude or the reversal potential for ISDN-mediated ΔI ($n = 5$; Fig. 2*B*).

In current clamp experiments we analysed the effects of ISDN on oocyte membrane potential and its interaction with the K^+ channel blocker Ba^{2+}. Under control conditions the membrane potential of *Xenopus* oocytes was −47·3 ± 2·3 mV ($n = 4$). Upon superfusion with ISDN

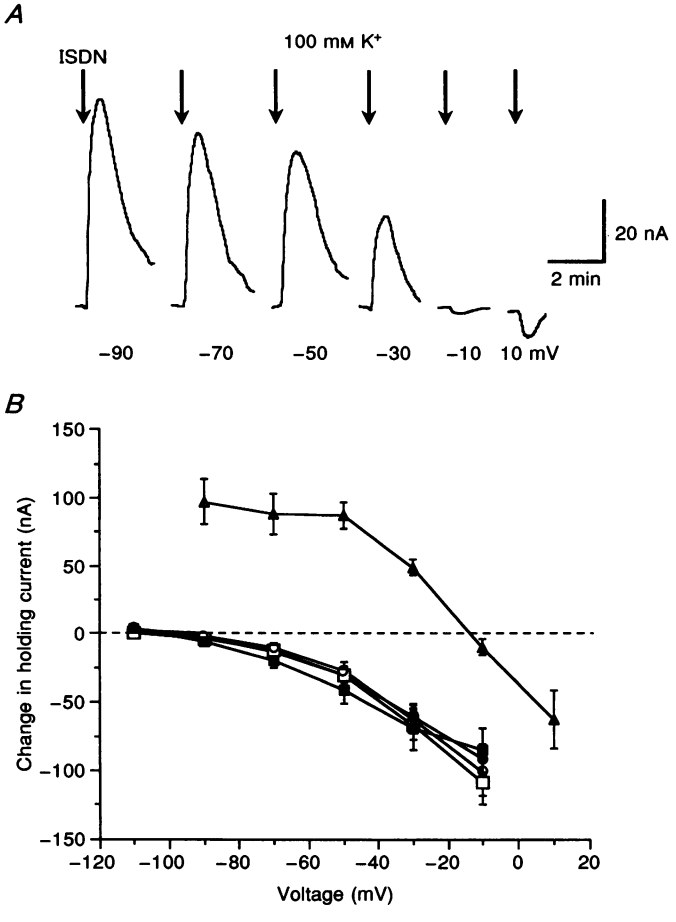

Figure 2. K^+ dependence of ΔI

A, *Xenopus* oocytes were voltage clamped at the indicated potentials. Extracellular Na^+ was replaced by K^+. The arrows indicate the start of a 30 s superfusion period with ISDN (1 mM). At 100 mM K^+ the reversal potential for ISDN-mediated effects is shifted to values around −10 mV. To clarify the effects of ISDN, the holding currents at the different potentials were aligned. *B*, arithmetic means (± S.E.M.) of the change in holding current caused by ISDN at extracellular solutions of: ●, control; □, 0 mM Ca^{2+}; ■, 0 mM Cl^-; ○, 10 mM Na^+; △, 100 mM K^+.

(1 mM) the cell depolarized by 14·5 ± 2·3 mV (Fig. 3*A*; $n = 4$). Ba^{2+} (superfused at 3 mM) caused a depolarization of the cell by 21·3 ± 1·8 mV ($n = 4$). However, ISDN and Ba^{2+} effects on oocyte membrane potential were not additive. Simultaneous superfusion of the oocyte with both ISDN and Ba^{2+} caused a depolarization of 23·3 ± 2·8 mV ($n = 4$), which was not significantly different from the depolarization mediated by Ba^{2+} alone. We found similar results in corresponding voltage clamp experiments at a holding potential of −50 mV. The ΔI induced by ISDN (1 mM) or Ba^{2+} (3 mM) was −23·1 ± 3·4 nA ($n = 4$) and −24·4 ± 3·2 nA ($n = 4$), respectively. When ISDN and Ba^{2+} were superfused together ΔI was −24·5 ± 3·2 nA (Fig. 3*B*; $n = 4$), which was not different from the effect of Ba^{2+} or ISDN alone, suggesting that Ba^{2+} and ISDN inhibit the same K^+ conductance. Subsequently, the concentration dependence of ISDN and Ba^{2+} effects on the holding current of oocytes at −50 mV was analysed. Both ISDN and Ba^{2+} exerted maximal effect on the holding current at approximately 3 mM with an apparent K_m of 0·21 ± 0·03 ($n = 6$) and 0·08 ± 0·01 mM ($n = 4$), respectively.

In another set of experiments we investigated the effects of ISMN and the nitrosothiol SNOC on the holding currents of *Xenopus* oocytes at −50 mV. In contrast to ISDN, both ISMN and SNOC had no effects on the holding currents ($n = 4$ for each compound). Furthermore, 1 mM $NaNO_3$ or $NaNO_2$ did not alter the holding current ($n = 4$ for each salt). Because NO is known to activate guanylate cyclase, the effects of ISDN on the holding current were analysed in the absence or presence of the guanylate cyclase inhibitor

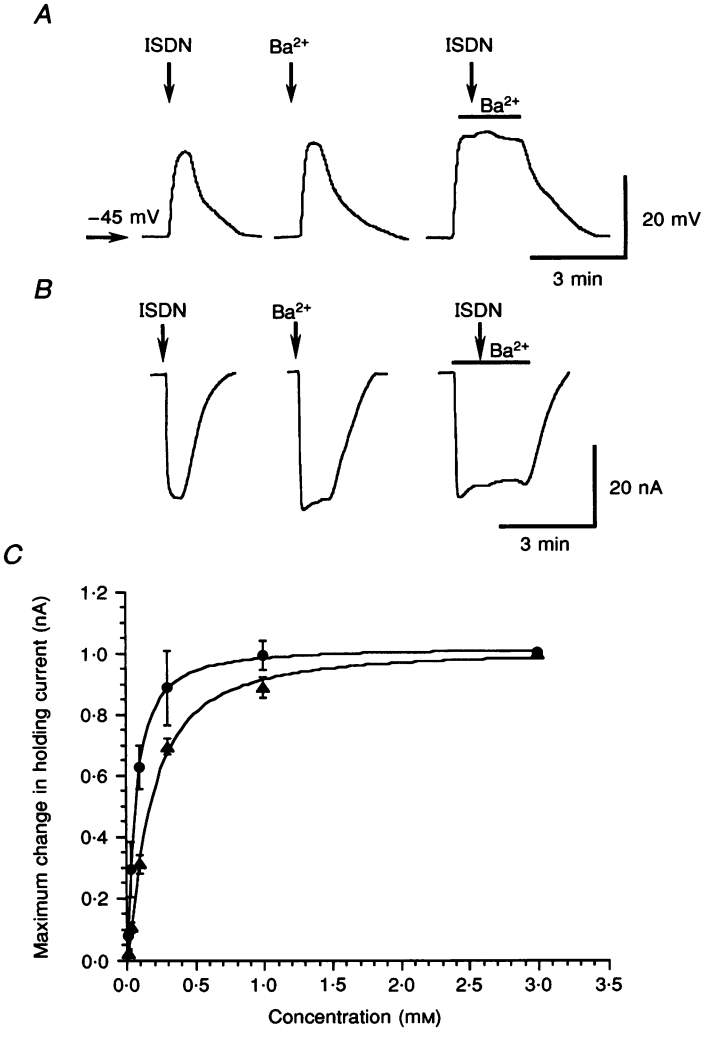

Figure 3. Effects of ISDN (1 mM) and Ba^{2+} (3 mM) on the oocyte membrane potential (*A*) or holding current at −50 mV (*B*)

Arrows indicate the start of a 30 s superfusion period. The horizontal arrow indicates a potential of −45 mV. When both Ba^{2+} and ISDN were applied together, the bar indicates the superfusion duration for Ba^{2+}. *C*, concentration dependence of Ba^{2+} (●) and ISDN (▲) effects on holding currents at −50 mV. The data were fitted to a Michaelis–Menten kinetic, K_m values were 0·08 ± 0·01 and 0·21 ± 0·03 mM for Ba^{2+} and ISDN, respectively.

LY 83583. LY 83583 (30 μM) did not inhibit the effects of ISDN. ΔI was $-15{\cdot}9 \pm 1{\cdot}6$ nA for ISDN (1 mM) alone and $-15{\cdot}6 \pm 1{\cdot}1$ nA when ISDN was applied in the presence of LY 83583 ($n = 4$).

Effects of ISDN on I_{sK}, Kv1.1 and IRK1 channels expressed in *Xenopus* oocytes

Finally, the effects of ISDN and ISMN were tested on the human slowly activating potassium channel, I_{sK} (Murai *et al.* 1988), the rat delayed rectifier channel, Kv1.1 (Christie *et al.* 1989), and the inward-rectifier channel, IRK1 (Fakler *et al.* 1994), which were expressed in oocytes after previous cRNA injection. Currents through slowly activating I_{sK} channels were induced with 15 s depolarizing voltage steps to -10 mV from a holding potential of -80 mV. At 1 mM, ISDN potently inhibited I_{sK}, while ISMN or $NaNO_3$ (both at 1 mM) had no significant effects (Fig. 4*A*; $n = 5$ and 4, respectively). The effects of ISDN on I_{sK} were concentration dependent with an apparent K_m of $0{\cdot}15 \pm 0{\cdot}10$ mM (Fig. 4*B*; $n = 4$).

K^+ outward currents through Kv1.1 were induced by 0·5 s voltage steps to -10 mV and K^+ inward currents through IRK1 were recorded after hyperpolarizing steps to -120 mV (the holding potential was -80 and -40 mV, respectively). In contrast to I_{sK}, ISDN (1 mM) had no appreciable effects on Kv1.1 and IRK1 ($n = 4$; Fig. 5*A–C*).

DISCUSSION

The aim of this study was to determine the effects of NO donors on K^+ channels either endogenously expressed in *Xenopus* oocytes or expressed after cRNA injection. The resting potential of defolliculated *Xenopus* oocytes is mainly determined by a permeability of the membrane to K^+ with a minor contribution of Na^+ and Cl^- fluxes (reviewed by Dascal, 1987). The results of this study show that indeed the membrane potential of defolliculated oocytes of *Xenopus laevis* is partially determined by an endogenous K^+ channel. This endogenous K^+ channel was inhibited by the pharmacologically exploited organic nitrate ISDN at commonly used concentrations (apparent K_m was approximately 0·2 mM). The K^+ channel blocker Ba^{2+} also inhibited a resting K^+ conductance with a similar K_m. Both ISDN and Ba^{2+} applied together have no additive effects on

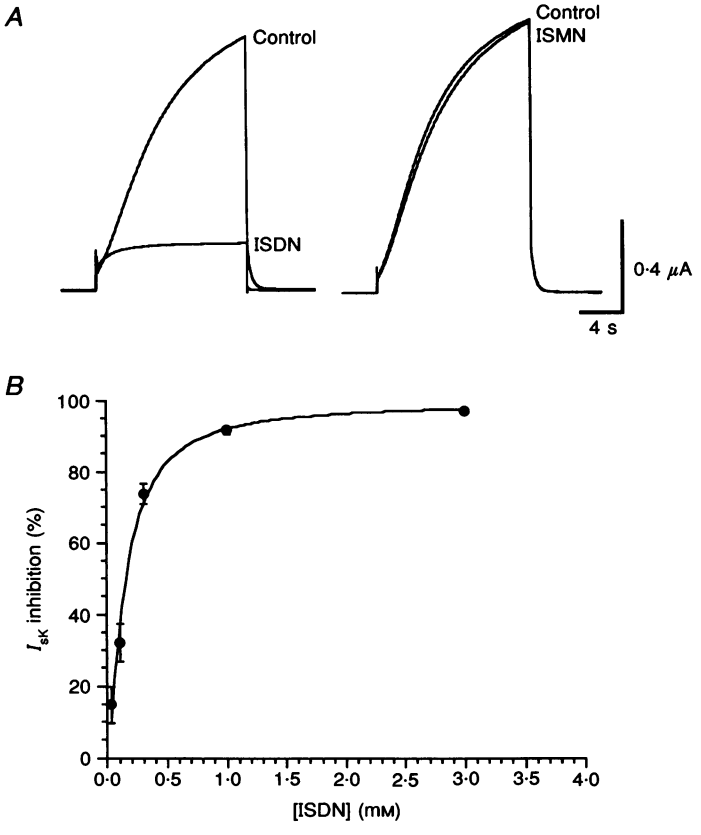

Figure 4. Effects of ISDN and ISMN (both at 1 mM) on I_{sK} channels expressed in *Xenopus* oocytes

I_{sK} was evoked with 15 s voltage steps to -10 mV from a holding potential of -80 mV every 45 s. At -10 mV ISDN decreases the endogenous K^+ conductance by approximately 50 nA, which is less than 5% of the measured I_{sK} amplitude. Effects of ISDN on the holding current are therefore not likely to contribute significantly to the effects seen on I_{sK}. *B*, concentration dependence of ISDN effects on I_{sK}. $K_m = 0{\cdot}15 \pm 0{\cdot}10$ mM.

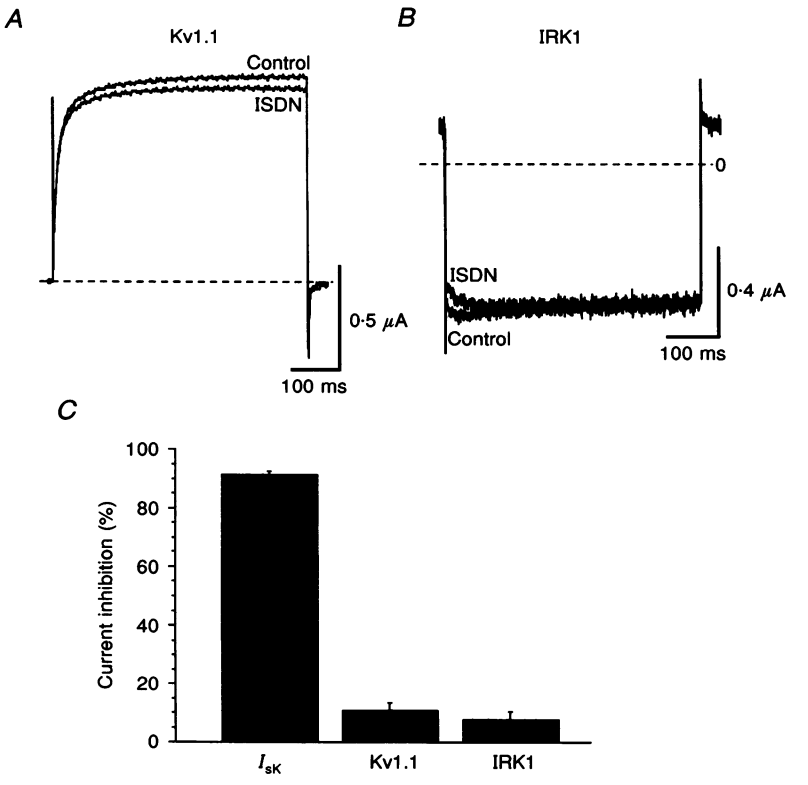

Figure 5. Effects of ISDN (1 mM) on Kv1.1 and IRK1 channels expressed in *Xenopus* oocytes

A, Kv1.1 channels were activated with 0·5 s depolarizing steps from −80 to −10 mV. *B*, inward K^+ currents through IRK1 channels were measured after 0·5 s hyperpolarizing voltage steps from −40 to −120 mV. The dashed lines indicate zero current. *C*, the histogram displays the inhibition of outward currents through I_{sK} and Kv1.1 channels and of inward currents through IRK1 channels by ISDN. The *Y*-axis gives the inhibition of the respective channels by 1 mM ISDN. Leakage currents are not subtracted.

the holding currents, suggesting that they inhibit the same K^+ conductance. ISDN-mediated alterations of the oocyte holding current reversed at approximately −100 mV which is the estimated equilibrium potential for K^+. Taking into account that the reversal potential for ISDN-mediated effects on oocyte holding current is dramatically shifted by changes in extracellular K^+, but not Ca^{2+}, Na^+ and Cl^-, ISDN appears to inhibit mainly a K^+ conductance.

The ISDN-sensitive K^+ conductance is slightly outwardly rectifying, but conducts K^+ in either direction around the equilibrium potential for K^+. A number of observations support the hypothesis that ISDN acts as a direct channel blocker rather than via metabolism to the second messenger NO. Firstly, the inhibition caused by ISDN is readily apparent upon superfusion and also promptly reversible upon washout. Secondly, other NO donors such as ISMN or SNOC do not affect the endogenous K^+ conductance. Thirdly, guanylate cyclase does not appear to be involved in K^+ channel inhibition, because inhibition of this enzyme did not alter ISDN-mediated K^+ channel inhibition.

Three major classes of K^+ channel-forming proteins have been characterized to date (for review see Catterall, 1994). The first two families are the voltage-gated delayed-rectifying K^+ channels (K_v, including the Ca^{2+}-activated maxi K^+ channel K_{Ca}) and the inwardly rectifying K^+ channels (K_{ir}, including G protein-activated K^+ channels) which share some similarities in the K^+ pore forming region. The third family of potassium channel proteins is represented by the I_{sK} protein (Takumi, Ohkubo & Nakanishi, 1988) which shares no common motif with the first two K^+ channel families. In this study we expressed one representative of each family and analysed the effects of ISDN on these K^+ channels. ISDN inhibited human I_{sK} channels with the same potency as the endogenous K^+ conductance without having appreciable effects on delayed-rectifying Kv1.1 and inwardly rectifying IRK1 channels. Moreover, ISMN, which unlike ISDN lacks the nitrate group in position 2 of the isosorbid molecule, did not inhibit I_{sK}, the same result as that observed for the endogenous K^+ conductance. Interestingly, the recently identified I_{sK} inhibitor azimilide (Busch, Malloy, Groh, Varnum, Adelman & Maylie, 1994) also inhibited the endogenous K^+ conductance without affecting Kv1.1 or IRK1 channels (data not shown). The identical pharmacological profile of I_{sK} channels expressed in *Xenopus* oocytes and endogenous

K^+ channels could therefore indicate their molecular relation. However, in this study only one representative of each of the K_v and K_{ir} families and no other ion channels, such as Ca^{2+} or Na^+ channels were studied for their sensitivity to ISDN, and effects of ISDN on other ion channels cannot be excluded. Similar to its effect on the endogenous K^+ conductance, ISDN appeared to inhibit I_{sK} via a direct mechanism. This is supported by our results with ISMN which, like ISDN, is a NO donor, but has no effects on I_{sK} expressed in *Xenopus* oocytes. Moreover, an NO-mediated positive regulation of I_{sK} after long-lasting superfusions (>10 min) with the NO donor *S*-nitroso-cystein was recently observed (Raber *et al.* 1995). However, there is an important difference between organic nitrates (such as ISDN) and nitrosothiols (such as SNOC). Specifically, while organic nitrates require a complex enzyme system for their metabolism to NO, NO release from nitrosothiols is spontaneous (see Feelisch & Noack, 1991). Therefore, the complete lack of I_{sK} regulation with ISMN indicates that organic nitrates cannot release NO in *Xenopus* oocytes.

In summary, this study describes direct effects of the organic nitrate ISDN on an endogenous K^+ conductance and I_{sK} channels expressed in *Xenopus* oocytes independent of NO. I_{sK} proteins in the heart represent the potassium conductance I_{Ks}, which may be involved in the generation of peroxide-induced arrhythmias (Cerbai, Ambrosio, Porciatti, Chiariello, Giotti. & Mugelli, 1991; Busch *et al.* 1995). Inhibition of I_{Ks} could possibly be the mechanism of antiarrhythmic action of novel antiarrhythmic compounds (Busch *et al.* 1994). Direct inhibition of I_{sK} channels by ISDN could therefore be of pharmacological interest.

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Acknowledgements

A. E. Busch and K. Kunzelmann are Heisenberg Fellows. I. Samarzija was sponsored by a Deutscher Akademischer Anstauschdienst fellowship. The work was supported by a grant from the Deutsche Forschungsgemeinschaft (Bu 704/3-1 to A. E.B.). The authors are indebted to Drs R. Swanson and J. Douglass for providing the I_{sK} and Kv1.1 clones, and to Drs P. Hausen and G. L. Busch for the discussion of the manuscript. We thank I. Struss and B. Noll for secretarial help and the preparation and handling of oocytes.

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Received 12 June 1995; accepted 21 September 1995.