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THE NITROSO-DONOR S-NITROSO-CYSTEINE REGULATES I_{sK} EXPRESSED IN *XENOPUS* OOCYTES VIA A c-GMP INDEPENDENT MECHANISM

Gertraud Raber, Siegfried Waldegger, Tobias Herzer, Erich Gulbins, Heini Murer*, Andreas E. Busch [1] and Florian Lang

Institute of Physiology, Eberhard-Karls-Universität Tübingen, Gmelinstr. 5, D-72076 Tübingen, Germany

*Institute of Physiology, University of Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland

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In *Xenopus* oocytes expressing slowly activating I_{sK} channels superfusion with the nitroso-donor S-Nitroso-Cysteine (SNOC) resulted in an increase of I_{sK}, which was greatly enhanced when the amino acid-exchanger rBAT was coexpressed. The effects of SNOC on I_{sK} could not be prevented by the guanylate cyclase inhibitor LY-83,583 and the cGMP kinase inhibitor H8, but was abolished in the presence of staurosporine. SNOC also increased the currents induced by the expression of protein mutants lacking intracellular sites, previously described to be involved in I_{sK} regulation by oxidation and phosphorylation. These data suggest that the NO-donor SNOC regulates I_{sK} indirectly via a cGMP independent, but staurosporine sensitive, pathway.

Nitric oxide (NO) is an important second messenger in a variety of tissues [1] and has been shown to exert its effects via cGMP synthesis [2] and/or direct oxidation of target proteins such as the NMDA receptor [3] and the calcium-activated K^+ channel of vascular smooth muscle [4]. NO has also been shown to be cardioprotective [5] and to control cardiac muscle cell function [6]. In heart, the I_{sK} protein underlies I_{Ks}, the slow component of voltage-activated K^+ current [7,8]. Moreover, I_{sK} channel blockade in heart by the novel class III antiarrhythmic Azimilide may play a therapeutical role in heart [9,10]. Expression of the I_{sK} protein [11] in *Xenopus* oocytes induces a very slowly activating K^+ current. The I_{sK} protein has been shown to be the target for I_{sK} regulation by peroxides [12] and protein kinase C mediated phosphorylation [13]. In the present study, we investigated a putative role of NO in I_{sK}

[1]To whom correspondence should be addressed. Fax: (49) (0) 7071-293 073. E-mail: abusch@mailserv.uni-tuebingen.de.

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regulation. For this purpose we tested the effects of the specific NO-donor S-Nitroso-Cysteine (SNOC) on oocytes expressing the I_{sK} protein either alone or together with rBAT, an amino acid transporter to facilitate SNOC transport into the oocytes.

MATERIALS AND METHODS

Handling and injection of *Xenopus* oocytes has been described previously in detail [14]. The two-microelectrode voltage clamp configuration was used to record currents from *Xenopus laevis* oocytes that were previously injected with cRNA transcribed in vitro from cDNA encoding for the I_{sK} protein from rat (r-I_{sK}) [11] and human (h-I_{sK}) [15]. Further several rat I_{sK} protein mutants, which have been described previously [16], were tested. I_{sK} was usually activated with 15 s voltage steps to -10 mV every 30 s from -80 mV holding potential. In some experiments the delayed rectifier K^+ channel Kv1.1 [17] and the Na^+-coupled inorganic phosphate transporter NaPi-2 [18] were expressed to test the effects of SNOC on these proteins. Kv1.1 was evoked with 500 ms voltage steps from -80 to -10 mV. Phosphate (P_i) induced currents (I_P) in oocytes expressing NaPi-2 were induced by superfusion of P_i (1 mM) at -50 mV as previously described [19]. Recordings were performed at 22° C using a Geneclamp amplifier (Axon Instruments, Foster City, USA) and MacLab D/A converter and software for data acquisition and analysis (ADInstruments, Castle Hill, Australia). If not otherwise stated, rBAT, which induces upon expression in oocytes electrogenic transport of neutral and dibasic amino acids [20], was coexpressed. The control solution contained (mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5 (pH 7.5). SNOC was synthesized directly before each experiment by mixing equimolar concentrations of L-cysteine and $NaNO_2$ at pH 2 and then added to the control solution to give in all experiments a final concentration of 1 mM. The extend of SNOC mediated I_{sK} increase varied considerably in different batches of oocytes, but could be observed in all oocytes tested (SNOC was tested in 15 different batches of oocytes). Effects of SNOC in the presence of distinct pharmacological agents or specific I_{sK} mutants were always compared to SNOC effects on a control group of 4 oocytes in each batch of oocytes. The microelectrodes were filled with 3 M KCl solution and had resistances between 0.5 to 1.1 MΩ. Chemicals were added from stock solutions into the superfusion solution as indicated. Chemicals used were $NaNO_2$, L-cysteine and staurosporine (Sigma) and LY-83,583 and H8 (RBI) and Di-butyryl-cGMP (Calbiochem). The recorded currents were leak-corrected and the amplitudes were measured at the end of the depolarizing voltage steps. To estimate activation kinetics (τ_{act}) of I_{sK}, a single exponential function was fitted to the current traces obtained at 0 and 20 mV. A single exponential function was also fitted to the tail currents at -80 mV to give a deactivation time constant (τ_{deact}). The conductance of oocytes was calculated by assuming an E_K of -100 mV. Data are presented as means with standard errors (S.E.M.), where **n** represents the number of experiments performed.

RESULTS AND DISCUSSION

Slowly activating, voltage-dependent human I_{sK} (h-I_{sK}) channels were expressed in *Xenopus* oocytes and induced the characteristically slowly activating outward currents upon depolarization. Superfusion of oocytes expressing h-I_{sK} with freshly synthesized SNOC (1 mM) induced a small increase in current amplitude of 14 ± 4.9 % and 15 ± 6.3 %, when r-I_{sK} and h-I_{sK} were expressed alone, respectively (n was 4 for each group). However, when h-I_{sK} was coexpressed with r-BAT, superfusion with SNOC increased h-I_{sK} by 93.2 ± 9.6 % (Fig. 1B; n=13). The effect occurred slowly and reached a maximum at about 15 minutes. The effect was also slowly reversible upon washout (to about 130 % of control amplitude after 20

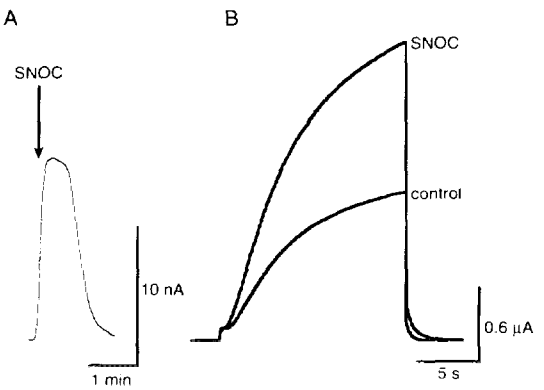

Fig. 1. Effects of SNOC (1 mM) on *Xenopus* oocytes expressing the amino acid transporter rBAT and h-I_{sK}. **A)** Superfusion with SNOC (1 mM) induces an outward current at -50 mV, similar to what was previously described for other neutral amino acids. The arrow indicates the start of a 20 s superfusion period with SNOC. **B)** SNOC increases I_{sK}. I_{sK} was evoked with a 15-s depolarizing step to -10 mV. The figure shows superimposed traces of h-I_{sK} under control and after 15 minutes under SNOC.

minutes washout). SNOC induced only in rBAT expressing oocytes at a holding potential of -50 mV an outward current (Fig. 1A), indicating its electrogenic transport as a neutral amino acid by rBAT into the oocyte, similar to what has been described for other neutral amino acids [20].

SNOC is known to release the membrane permeable Nitric oxide (NO). The subsequently observed large increase of I_{sK} in oocytes coexpressing rBAT is therefore presumably the consequence of higher intracellular concentrations of SNOC and consequently NO compared to oocytes not expressing an amino acid transporter. As the effects of SNOC were observed over a time range, where SNOC itself underwent already redox-reactions (half lifetime is about 20 minutes), the effects appeared to be the consequence of NO release.

In oocytes expressing rBAT in addition to the I_{sK} protein, L-cysteine (1 mM) induced at -50 mV an outward current similar to SNOC; however, L-cysteine (1 mM) or $NaNO_2$ (1 mM) exerted no effects on I_{sK}, nor was an effect seen with inactivated SNOC (SNOC was used 1 week after synthesis; n=4, 4 and 5, respectively; data not shown). These results suggest that the effects of SNOC on I_{sK} are indeed NO-mediated, because none of the other possible reactive agents affected I_{sK}.

In another set of experiments the effects of SNOC were tested on K^+ channel Kv1.1 [17] and the renal Na^+ coupled P_i transporter NaPi-2 [18]. SNOC did not alter the voltage-activated K^+ currents through Kv1.1 nor did SNOC alter the P_i induced currents through NaPi-2 (data not shown; n was 5 and 5, respectively).

The effects of SNOC on h-I_{sK} conductance-voltage relationship, activation- and deactivation kinetics were analyzed in detail. Performing 15 s voltage steps from -40 to 20 mV (increment 20 mV) the voltage needed to evoke halfmaximal conductance ($V_{0.5}$) was shifted from -5.9 ± 0.6 mV under control (n=5) to -20.2 ± 0.1 mV after SNOC (Fig. 2A, B; n=5). SNOC did not significantly alter τ_{act} at 0 and 20 mV (Fig. 3A; n=5), but increased τ_{deact} (Fig. 3B; n=5). This

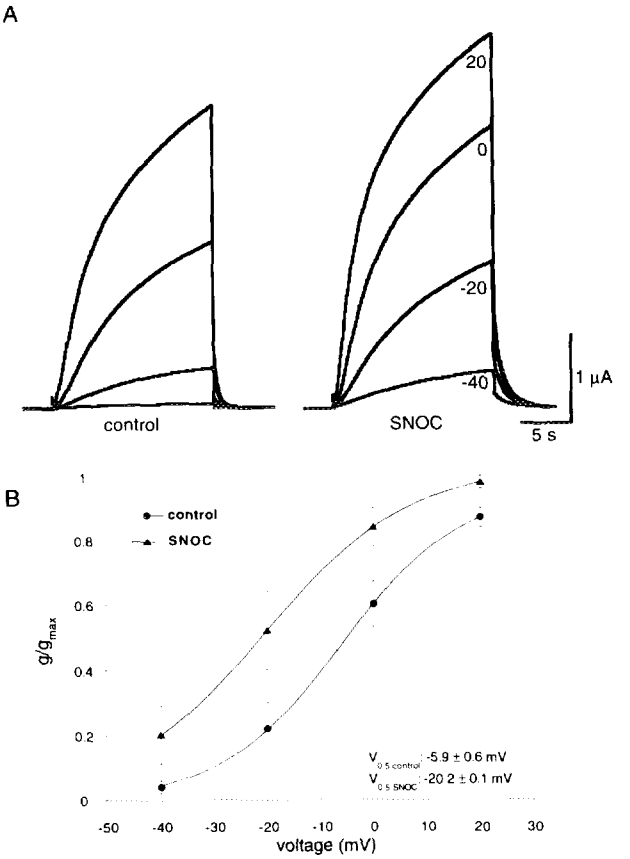

Fig. 2. Effects of SNOC on current-voltage relationship of h-I_{sK}. **A)** h-I_{sK} was evoked with 15-s voltage steps every 45 s to the potentials indicated. Under SNOC h-I_{sK} is increased at all potentials tested. **B)** Conductance-voltage relationship of h-I_{sK} under control and SNOC. The calculated conductances were normalized against the maximal conductance observed in the individual oocytes. Data represent means ± S.E.M.

result suggests that SNOC regulation mediates a stabilization of the open state of I_{sK}, because deactivation occurs more slowly.

Previously, r-I_{sK} was shown to be regulated by oxidation of Cys 107 [12] and protein kinase C mediated phosphorylation of Ser 103 [13]. Moreover, kinase A activation increases r-I_{sK} by an undefined mechanism [21]. Because NO has been shown to regulate a number of proteins by oxidation or phosphorylation via cGMP-dependent kinase, we tested SNOC on r-I_{sK} protein mutants, in which the intracellular Cys 107 was mutated to a Ser (r-I_{sK} C107S) or which lacked the extracellular domain 10-39 and the intracellular protein domain from residue Ala 94 to the protein end (r-I_{sK} del 10-39, A94). These protein mutants induced control currents with the same general properties as the wild-type protein. However, SNOC superfusion increased both r-I_{sK} C107S and r-I_{sK} del 10-39, A94 by 40.2 ± 11.4% (n=5) and 50.4 ± 5.8 %, respectively. Oxidation of the r-I_{sK} protein at Cys 107 or a putative NO induced phosphorylation of the r-I_{sK} protein itself seems therefore unlikely as an explanation for the SNOC mediated effects.

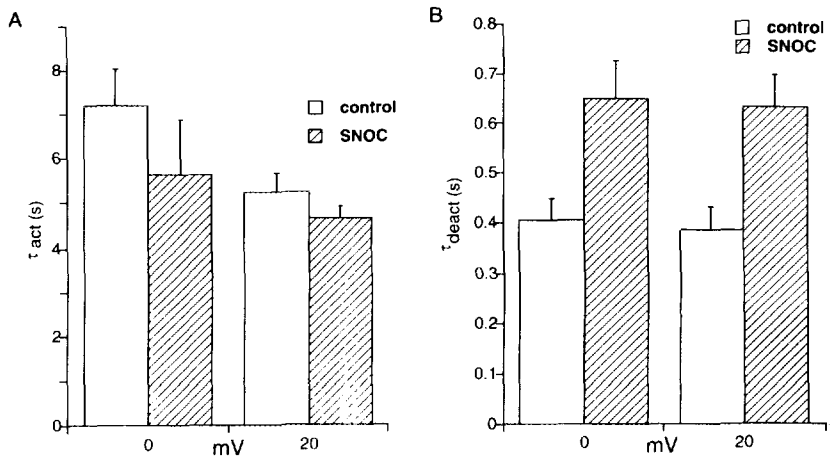

Fig. 3. Effects of SNOC on rate of h-I_{sK} activation and deactivation. **A)** Under SNOC the estimated activation time constants (τ_{act}) at 0 and 20 mV were not significantly different. **B)** SNOC significantly increased the estimate time constants for deactivation (τ_{deact}) . τ_{deact} was determined at -80 mV after 15-s depolarizing steps to 0 and 20 mV. Data represent means ± S.E.M.

NO is known for inducing the synthesis of cGMP and a subsequent activation of cGMP dependent kinase [2]. Therefore, SNOC was tested in the presence of the guanylate cyclase inhibitor LY-83,583 (30 μM) and the cGMP dependent kinase inhibitor H8 (3 μM). LY-83,583 and H8 both inhibited h-I_{sK} by -14.9 ± 1.3% and -13.2 ± 2.4%, respectively. However, superfusion with SNOC increased h-I_{sK} also in the presence of LY-83,583 and H8 (Fig. 4A) by 49.0 ± 5.9 % (n=7) and 74.6 ± 19.8 % (n=7), respectively. Direct activation of cGMP dependent kinase with the membrane-permeable Di-butyryl-cGMP (4 mM) did not alter h-I_{sK} (n=5; data not shown). The mechanism involved in the increase of I_{sK} caused by SNOC appears therefore independent of cGMP production or activation of cGMP-dependent kinase.

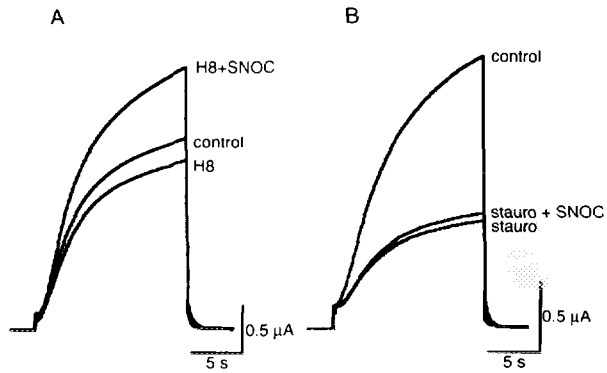

Fig. 4. Effects of H8 (3 mM) and staurosporine (stauro; 1 μM) on h-I_{sK} and SNOC-mediated h-I_{sK} regulation. **A)** H8 (3 μM) slightly inhibits h-I_{sK}, but does not prevent SNOC-mediated positive regulation. **B)** Staurosporine inhibits h-I_{sK} and greatly suppresses h-I_{sK} regulation by SNOC.

Furthermore, we tested the effects of the unspecific kinase inhibitor staurosporine on SNOC mediated I_{sK} regulation. Staurosporine (1 μM) alone reduced h-I_{sK} by -65.7 ± 4.3 % (n= 7). Moreover, staurosporine greatly inhibited the SNOC mediated h-I_{sK} increase (Fig. 4B). In the presence of staurosporine, SNOC increased h-I_{sK} by 11.4 ± 5.5 % (n=7), which was significantly less than in the absence of staurosporine (SNOC increased h-I_{sK} of the control group by 80.8 ± 22.5 %, n=4). The strong inhibition of h-I_{sK} by staurosporine alone suggests a h-I_{sK} regulation by phosphorylation already under control conditions.

In summary, the data presented in this study suggest a role for NO in I_{sK} regulation. A regulation of the I_{sK} protein in heart by NO could be involved in the regulatory effects seen for NO in heart tissue [5,6]. SNOC did not exert an effect on I_{sK} in the presence of the kinase inhibitor staurosporine suggesting that phosphorylation via an unidentified kinase is involved in I_{sK} regulation. However, phosphorylation does not seem to take place at the I_{sK} protein itself, because I_{sK} mutants lacking intracellular phosphorylation sites were still regulated by SNOC, suggesting that NO acts indirectly. However, the definite mechanism of I_{sK} regulation and the type of kinase activated by SNOC remains to be clarified.

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REFERENCES

1. Moncada, S. and Palmer, R.M.J. (1991) TIPS 12, 130-131.
2. Furchgott, R. F. (1990) Acta Physiol. Scand. 139, 257-270.
3. Lei, S.Z., Pan, Z.H., Aggarwal, S.A., Chen, H.V., Hartman, J., Sucher, N.J. and Lipton, S.A. (1992) Neuron 1992, 1087-1099.
4. Bolotina, V.M., Njibi, S., Palaino, J.J., Pagano P.J. and Cohen, R.A. (1994) Nature 368, 850-853.
5. Lefer, D.J., Nakanishi, K., Johnston, W.E. and Vinten-Johansen, J. (1993) Circulation 88, 2337-2350.
6. Balligand, J.L., Kelly, R.A., Marsden, P.A., Smith, T.W. and Michel, T. (1993) Proc. Natl. Acad. Sci. USA 90, 347-351.
7. Honore, E., Attali, B., Romey, G., Heurteaux, C., Ricard, P., Lesage, F. and Lazdunski,M. (1991) EMBO J 10, 2805-2811.
8. Varnum, M.D., Busch, A.E., Bond, C.T., Maylie, J. and Adelman, J.P. (1993) Proc. Natl. Acad. Sci. USA 90, 11528-11532.
9. Busch, A.E., Malloy, K.J., Varnum, M.D., Adelman, J.P., North, R.A. and Maylie,J. (1994) Biochem. Biophys. Res. Comm. 202, 265-270.
10. Busch, A.E., Herzer, T., Takumi, T., Krippeit-Drews, P., Waldegger, S. and Lang, F. (1994) Eur. J. Pharmacol. 264, 33-37.
11. Takumi, T., Ohkubo, H. and Nakanishi, S. (1988) Science 242, 1042-1045.
12. Busch, A.E., Herzer, T., Waldegger, S., Wagner, C.A., Gulbins, E., Takumi, T., Moriyoshi, K., Nakanishi, S. und Lang, F. (1995) Biophys. J. (abstract; in press).
13. Busch, A.E., Varnum, M.D., North, R.A. and Adelman, J.P. (1992) Science 255, 17075-1707.
14. Busch, A.E., Kavanaugh, M.P., Varnum, M.D., Adelman, J.P.and North, R.A. (1992) J. Physiol. 450, 491-502.

15. Murai, T., Kakizuka, A., Takumi, T., Ohkubo, H. and Nakanishi, S. (1989) Biochem. Biophys. Res. Commun. 161, 176-181.
16. Takumi, T., Moriyoshi, K., Aramori, I., Ishii, T., Oiki, S., Okada, Y., Ohkubo, H. and Nakanishi, S. (1991) J. Biol. Chem. 266, 22192-22198.
17. Christie, M.J., Adelman, J.P., Douglass, J. and North, R.A. (1989) Science 244, 221 - 224.
18. Magagnin, S., Werner, A., Markovich, D., Sorribas, V., Stange, G., Biber, J. and Murer, H. (1993) Proc. Natl. Acad. Sci. USA 90, 5979 - 5983.
19. Busch, A.E., Waldegger, S., Herzer, T., Biber, J., Markovich, D., Murer, H. and Lang, F. (1994) Proc. Natl. Acad. Sci. USA 91 (17), 8205-8208.
20. Busch, A.E., Herzer, T., Waldegger, S., Schmidt, F., Palacin, M., Biber, J., Markovich, D., Murer, H. and Lang, F. (1994) J. Biol. Chem. 269, 25581-25586.
21. Blumenthal, E.M. and Kaczmarek, L.K. (1992) J. Neurosci. 12, 290-296.