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Short communication

Reduction of hERG potassium currents by hyperosmolar solutions

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Abstract

We investigated the effects of hyperosmolar solutions on human ether-a-go-go related gene (hERG) potassium currents in chinese hamster ovary (CHO) cells. The addition of D-mannitol to the external solution caused cell shrinkage and reduced current amplitude. The effects were at least partially reversible. Exposure to 108 mM mannitol decreased current amplitude by 57±13%. Major effects on current–voltage relations were not observed. Exposure to 308 mM mannitol reduced the current by 89±5%, i.e. comparable to the block induced by 1 μM of the selective hERG channel blocker E-4031. We conclude that the investigation of hyperosmolar drug formulations requires control solutions of comparable osmolarity to separate specific drug effects from non-specific effects of hyperosmolarity.

Keywords: Patch-clamp technique; Human ether-a-go-go related gene; hERG; CHO cells; D-mannitol; Hyperosmolar solution

1. Introduction

Inhibition of the repolarizing delayed rectifier current I_{Kr} is regarded as the major mechanism for drug-induced QT-interval prolongation of the electrocardiogram. The prolongation of the QT-interval is associated with an increased risk of fatal arrhythmia. I_{Kr} is conducted by human ether-a-go-go related gene (hERG) channels (Sanguinetti et al., 1995), and the absence of significant hERG channel inhibition at therapeutic drug concentrations is an important prerequisite for development of a new drug.

Several expression systems, such as transfected *Xenopus* oocytes, chinese hamster ovary (CHO) cells and human embryonic kidney cells (HEK293), are available to evaluate the hERG channel blocking properties of novel drug development candidates. These expression systems allow the investigation of hERG currents in the absence of other contaminating currents. The models are well characterized, and the effects of temperature (Vandenberg et al., 2006), extracellular pH (Jo et al., 1999) and of other biophysical parameters on hERG currents have been described.

Some development compounds are intended to be clinically applied as highly concentrated solutions, e.g. contrast agents for diagnostic imaging. Therefore, safety pharmacology studies occasionally include the investigation of hERG current inhibition at compound concentrations in the high millimolar range. Under these conditions, the increase of drug concentration in the bath solution is paralleled by a significant increase of extracellular osmolarity. As a result it may be difficult to separate compound-specific pharmacological effects from unspecific effects due to the hyperosmolar condition. In the present work we focussed on osmolarity effects using D-mannitol as an osmoactive compound with no or little direct pharmacological action. We studied the effects of hyperosmolar external solutions on the amplitude, voltage-dependence and kinetics of currents conducted by hERG channels stably expressed in CHO cells.

2. Materials and methods

CHO cells stably expressing the hERG gene were generated using a pcDNA3-KCNH2-FLAG expression vector (Protinac GmbH, Hamburg, Germany). Cells were passaged at 37 °C and

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5% CO_2 atmosphere in 25 cm^2 cell culture flasks (Corning Inc., Corning, NY, USA), containing 5 ml MEM alpha medium supplemented with 10% (v/v) heat inactivated fetal calf serum, 1% (v/v) penicillin/streptomycin/glutamin-solution and 200 μg/ml geneticin G-418 sulfate (all from Gibco BRL, Eggerstein, Germany). For electrophysiological recordings, submaximally confluent cells were used after 1–3 days of culture at a seeding density of $1–4 \times 10^5$ cells per flask. The cells were scraped off from the flask and transferred to the recording chamber.

Membrane currents were measured in the whole cell configuration of the patch clamp technique at room temperature (Hamill et al., 1981). For electrophysiological recordings the cells were transferred to a recording chamber (RC-24E, Warner Instruments, Hamden, CT, USA) and allowed to attach to the glass bottom for approximately 15 min. Subsequently, the bath was perfused continuously at a rate of 0.6 ml/min. External solutions of different osmolarities and E-4031 were applied using a rapid solution exchanger (DAD-12 superfusion system, ALA Scientific Instruments, Westbury, NY, USA). Membrane currents were measured with a List EPC-7 amplifier (HEKA, Lamprecht/Pfalz, Germany) under the control of ISO-2 software (MFK, Niedernhausen, Germany). Patch electrodes were pulled with a horizontal puller (P-97, Sutter Instrument Company, Navato, CA, USA) from filamented borosilicate glass capillaries (Hilgenberg GmbH, Malsfeld, Germany). The tip resistance was 3–6 MΩ, when filled with electrode solution. Membrane currents were low-pass filtered at 3 kHz. For the investigation of hERG-encoded potassium channels CHO cells were clamped at a holding potential of −80 mV. Test pulses were applied every 10 s. For the time courses and for the osmolality–response curve currents were elicited during a 1 s step to +20 mV, and current amplitude was measured as the late current at the end of a 1 s step to −40 mV. Other protocols are described in the legends of the respective figures. The osmolality of solutions (in mOsm/kg) was assessed by means of a vapor pressure osmometer (Vapro 5520, Wescor Inc., Utah, USA). The electrode solution had the following composition (mM): KCl 130, EGTA 1, Mg-ATP 2, glucose 5, HEPES 10. The pH was adjusted to 7.2 using KOH. The measured osmolality was 299±3 mOsm/kg. The standard external solution had the following composition (mM): NaCl 130, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1, glucose 5, and HEPES 10. The pH was adjusted to 7.4 using NaOH. The measured osmolality of this solution was 273±3 mOsm/kg. We used D-mannitol as an osmoactive compound assuming that there are no compound-specific effects on hERG channels even at concentrations in the high mM range. D-Mannitol was obtained from Sigma-Aldrich (St. Louis, MO, USA) and E-4031 from Calbiochem (San Diego, CA, USA).

3. Results

Every experiment started with a 5–10 min period of current stabilization in standard external bath solution. Subsequent exposure of the cells to hyperosmolar external solutions reduced current amplitude within a few minutes. Fig. 1 demonstrates time courses of three representative cells with exposure to 108 mM (A), 208 mM (B) and 308 mM (C) D-mannitol, respectively. Original current traces from the cell exposed to

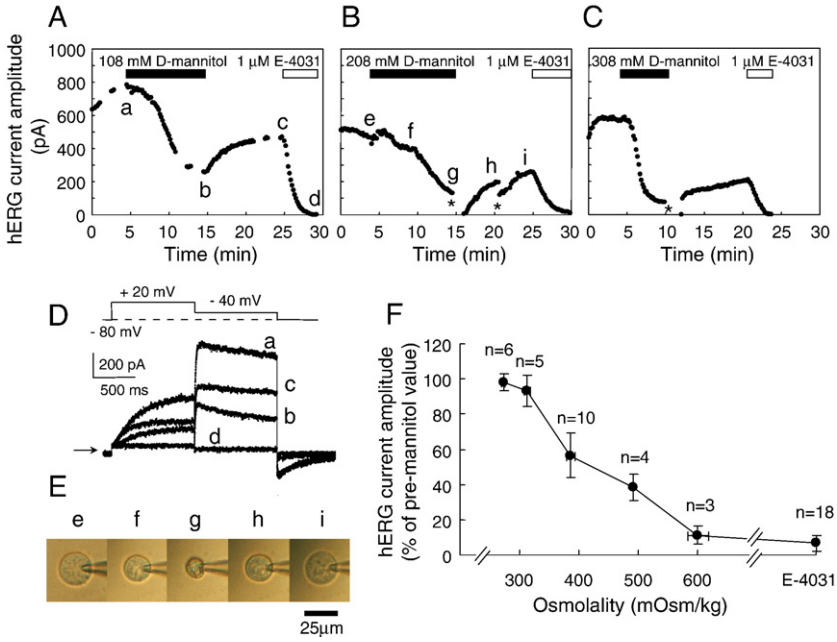

Fig. 1. Effects of hyperosmolar solutions on hERG-associated currents and cell morphology. Time courses shown in the top row demonstrate the effects of increasing external osmolality from 273±3 mOsm/kg (standard solution) by exposure to (A) 108 mM mannitol (387±7 mOsm/kg), (B) to 208 mM mannitol (491 mOsm/kg), and (C) to 308 mM mannitol (601±18 mOsm/kg), respectively. The effects were partially reversible. Asterisks indicate instable recording conditions with transient leak. E-4031 (1 μM) applied at the end of each experiment almost completely blocked the outward current. (D) Original current traces recorded from the cell exposed to 108 mM mannitol. The arrow indicates zero current. (E) Cell morphology before, during and after exposure to 208 mM mannitol. Original current traces and photographs refer to time courses in (A) and (B), respectively, with time points indicated by small letters. (F) Osmolality–current relationship (mean values±standard deviation).

108 mM mannitol are shown in Fig. 1D. The reduction of hERG current was paralleled by cell shrinkage as depicted in Fig. 1E for the cell exposed to 208 mM mannitol. Both effects, i.e. the reduction of current amplitude and cell shrinkage, were at least partially reversible upon switching back to standard external solution. At the end of each experiment the cells were exposed to 1 μM E-4031, a selective blocker of hERG K^+ currents, which almost completely blocked the outward current. An osmolality–response curve is shown in Fig. 1F. At 108 mM mannitol the current was reduced by 57±13% (n=10). The highest mannitol concentration tested (308 mM) reduced the hERG current to a similar extent as observed in presence of 1 μM E-4031 after washout of mannitol.

In three experiments the cells were exposed to 208 mM mannitol in the presence of 1 μM E-4031 to investigate, whether the hyperosmolar condition would evoke a previously silent current. This was, however, not the case, as the different voltage-protocols did not elicit any inward or outward current under conditions, where hERG channels were completely blocked (data not shown).

With regard to voltage-dependence the effects of 108 mM mannitol were investigated on current–voltage relation, voltage-dependence of current activation and instantaneous current–voltage relation. The current–voltage relations before, during and after exposure to 108 mM mannitol were established by measuring late-current amplitude during the first step of the two step protocol depicted on top of Fig. 2A, they are shown in Fig. 2B. The activation curves (Fig. 2C) were constructed by measuring the peak current amplitude during the second step of the voltage protocol (−60 mV). The current–voltage relation and activation curves indicate that the reduction of current amplitude is not associated with major shifts in voltage-dependence. If at all, there was a slight trend towards activation at more negative potentials after washout of mannitol. The reversal potential of the instantaneous current, i.e. peak current during the repolarizing second step of the voltage-protocol shown on top of Fig. 2D, was comparable before, during and after exposure of the cells to 108 mM mannitol (Fig. 2E).

Finally, we assessed the effects of hyperosmolar solutions on the kinetics of current activation and deactivation (n=4). Exposure to 108 mM mannitol had no significant effect on activation kinetics at 0 mV (time constant = 1000±389 ms under control conditions vs. 805±190 ms during exposure to mannitol; mean value±standard deviation) and at +20 mV (285±88.4 ms vs. 282±95.0 ms), the potentials at which the maximal current amplitudes were observed. With regard to current deactivation at −60 mV, the exposure to mannitol reversibly accelerated the deactivation kinetics, as shown in Fig. 2A for currents previously activated at 0 mV and at +40 mV (compare current traces at −60 mV in “a” and “b”). The

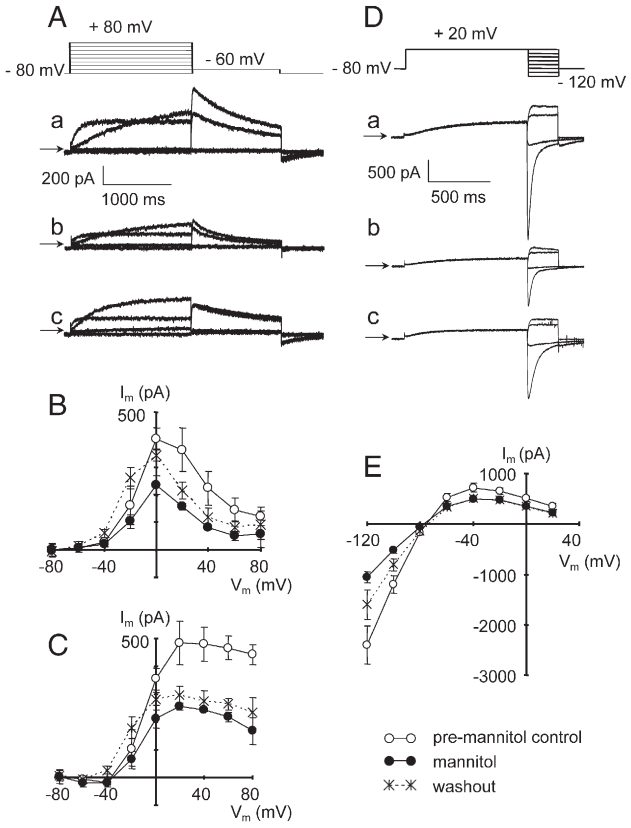

Fig. 2. Voltage-dependence of hERG current before, during and after exposure to 108 mM mannitol. (A) Original current traces at −80, −40, 0, and +40 mV are provided for time-points corresponding to the time-course in Fig. 1A as indicated by small letters. (B) Current–voltage relationship of activating current and (C) activation curve determined from the deactivating tail current (peak current). (D) Original current traces at −120, −80, −40, and 0 mV are provided for time-points corresponding to the time-course in Fig. 1A as indicated by small letters. (E) Instantaneous current–voltage relationships of peak tail currents. Mean values±standard deviation of n=4 cells.

deactivation kinetics following current activation at +20 mV (traces not shown) were significantly accelerated: in the presence of mannitol the time constant was 509±61.8 ms as compared to 718±41.9 ms under control conditions ($p=0.03$, paired *t*-test). The kinetics of the deactivating tail inward current at −120 mV (Fig. 2D) during exposure to mannitol (39.5±14.0 ms), however, were not significantly different from deactivation under control conditions (35.5±7.14 ms).

4. Discussion

In the present work we demonstrate that increased extracellular osmolarity is associated with reduction of hERG currents and cell shrinkage in CHO cells stably expressing hERG channels. Major effects on voltage-dependence were not observed.

The response to hypertonic or hypotonic conditions involves several well-balanced mechanisms that act in concert, and they include ion flux through ion channels (Lang et al., 1998). In comparison to somatic cells, e.g. cardiomyocytes, the repertoire of regulatory options appears to be limited in the expression system used for the present work. Under conditions, where hERG channels were blocked by E-4031, there was no other ion current detectable in response to hyperosmolarity, indicating that other channels, e.g. a chloride-dependent cation conductance or any other endogenous background current, are not available or not evoked by exposure to hyperosmolar solutions. The osmotic loss of cell water without significant transition of ions through the membrane should theoretically increase the ion concentrations within the cell. A putative increase of the intracellular K^+ concentration would enhance the concentration gradient between the intracellular and extracellular space and lower the K^+ equilibrium potential. Given that ion selectivity of the hERG channel remains preserved during exposure to hyperosmolar solutions, the more negative K^+ equilibrium potential should be reflected by a shift of the reversal potential towards more negative potentials. However, a clear negative shift was not observed (Fig. 2E). This can either mean, that the increase of the intracellular K^+ concentration was only mild, or that a negative shift of the reversal potential was overlaid by a positive shift due to loss of K^+ selectivity of the hERG channel.

The mechanisms involved in hERG current reduction during exposure to hyperosmolar solutions in the CHO expression system remain speculative. An increase of the K^+ concentration gradient, in general, should enhance the driving force for diffusion of K^+ through potassium channels, leading to higher outward current amplitudes. On the other hand, it is a characteristic property of the hERG channel, that current amplitude is reduced in response to lowering the extracellular K^+ concentration (Sanguinetti et al., 1995), i.e. increasing the transmembrane K^+ concentration gradient, although a putative increase of the intracellular K^+ concentration and the reduction of extracellular K^+ concentrations may affect hERG channel properties in different ways. The reduced hERG current amplitude observed in the present work may have other reasons as well: in a simple mechanistic model, cellular shrinkage causes plication of the plasma membrane, and this may be associated with reduced numbers of hERG channels being available for K^+ conductance. Furthermore, single channel conductance may be reduced by hyperosmolar solutions as seen with another type of K^+ channel, the Shaker B channel (Starkus et al., 1995).

The observed association between hyperosmolar conditions and reduced hERG currents may be limited to the artificial expression system. It is not consistent with the reduced I_{Kr} in guinea-pig ventricular myocytes exposed to *hypoosmolar* solutions (Rees et al., 1995). Therefore, one has to be careful when transferring the findings presented here to somatic cells, where usually several mechanisms of osmoregulation are available and different kinds of ion channels are coexpressed.

With all the limitations discussed above our findings have practical implications. The assessment of new drug candidates in the hERG channel assay is an important component of the preclinical drug development process. Whenever compounds are tested as hyperosmolar solutions, the osmolarity-induced changes of cell morphology and of hERG current properties have to be considered. For those cases, where hyperosmolarity of the test solution is caused by the solvent, e.g. dimethyl sulfoxide (DMSO), the proposed method of adding an equivalent solvent concentration to the pipette solution may be an approach to prevent cell shrinkage (Du et al., 2006). However, if hyperosmolarity of the external solution results from the test compound itself, it is essential to investigate control solutions of comparable osmolarity in parallel to be able to differentiate between compound-specific effects and unspecific effects due to hyperosmolarity.

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