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Activation of the human intermediate-conductance Ca^{2+}-activated K^+ channel by 1-ethyl-2-benzimidazolinone is strongly Ca^{2+}-dependent

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Abstract

Modulation of the cloned human intermediate-conductance Ca^{2+}-activated K^+ channel (hIK) by the compound 1-ethyl-2-benzimidazolinone (EBIO) was studied by patch-clamp technique using human embryonic kidney cells (HEK 293) stably expressing the hIK channels. In whole-cell studies, intracellular concentrations of free Ca^{2+} were systematically varied, by buffering the pipette solutions. In voltage-clamp, the hIK specific currents increased gradually from 0 to $\approx$300 pA/pF without reaching saturation even at the highest Ca^{2+} concentration tested (300 nM). In the presence of EBIO (100 μM), the Ca^{2+}-activation curve was shifted leftwards, and maximal currents were attained at 100 nM Ca^{2+}. In current-clamp, steeply Ca^{2+}-dependent membrane potentials were recorded and the cells gradually hyperpolarised from −20 to −85 mV when Ca^{2+} was augmented from 0 to 300 nM. EBIO strongly hyperpolarised cells buffered at intermediate Ca^{2+} concentrations. In contrast, no effects were detected either below 10 nM (no basic channel activation) or at 300 nM Ca^{2+} (V_m close to E_K). Without Ca^{2+}, EBIO-induced hyperpolarisations were not obtainable, indicating an obligatory Ca^{2+}-dependent mechanism of action. When applied to inside-out patches, EBIO exerted a Ca^{2+}-dependent increase in the single-channel open-state probability, showing that the compound modulates hIK channels by a direct action on the α-subunit or on a closely associated protein. In conclusion, EBIO activates hIK channels in whole-cell and inside-out patches by a direct mechanism, which requires the presence of internal Ca^{2+}.

Keywords: Ca^{2+}-activated K^+ channel; 1-Ethyl-2-benzimidazolinone; Patch-clamp; Ca^{2+}-dependency

1. Introduction

Ca^{2+}-activated K^+ channels are almost ubiquitously expressed in mammalian cells, and they link the second messenger systems involving Ca^{2+} to the electrical activity of cells. Three major subtypes of Ca^{2+}-activated K^+ channels exist: large-conductance (BK), intermediate-conductance (IK), and small-conductance (SK) channels, which, apart from their unit

Abbreviations: hIk, human intermediate-conductance Ca^{2+}-activated K^+ channel; EBIO, 1-ethyl-2-benzimidazolinone; CLT, clotrimazole; HEPES, *N*-Hydroxyethylpiperazine-*N*′-ethanesulfonic acid; EGTA, ethyleneglycol-bis-(β-amino-ethylether) N,N′-tetra-acetic acid; DMSO, dimethylsulfoxide; FCS, foetal calf serum; DMEM, Dulbecco's modified Eagle's Media; HEK 293, human embryonic kidney cells; V_m, membrane potential; E_K, K^+ equilibrium potential; R_S, series resistance

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conductances, also differ in primary structure, tissue distribution, voltage-dependency, and Ca^{2+}-sensitivity. Furthermore, each channel subtype has a distinct pharmacology (for a review, see [1]). The classical selective blockers of BK and some SK channels are the peptides iberiotoxin and apamin, respectively, whereas the antifungal compound clotrimazole selectively blocks IK channels. Compounds, which activate BK channels have been reported, and these include the plant alkaloid dihydrosoyasaponin [2] and the substituted benzimidazolone NS1619 [3]. 1-Ethyl-2-benzimidazolinone (EBIO) has been demonstrated to activate the basolateral Ca^{2+}-activated K^+-conductance in colonic T-84-derived epithelia [4], a conductance entirely attributable to IK channels. The compound also activates the recently cloned hIK channel [5]. Activation of the basolateral Ca^{2+}-activated K^+-conductance is the primary mechanism for stimulation of transepithelial Cl^- secretion by Ca^{2+}-raising secretagogues such as acetylcholine. Generally, in secretory epithelia activation of the basolateral K^+ conductances in parallel with the luminal cAMP-stimulated Cl^- conductance counteract the depolarising effect of Cl^- efflux and therefore effectively maintain the driving force on Cl^- during sustained secretion. Therapeutically, activation of basolateral K^+ conductances may even be considered as a mechanism for partial normalisation of secretory responses in epithelial tissue defective in CFTR [6]. Recently, clotrimazole has been shown to inhibit activated secretion in T-84 epithelia as well as cholera toxin-induced diarrhoea in mice, thus demonstrating a key function of hIK channels in colonic hypersecretory responses [7,8]. Clotrimazole has also been demonstrated to reduce erythrocyte dehydration in sickle cell patients [9] implicating a critical role of the IK channel in the pathogenesis of sickle cell disease.

In the present study, we have investigated the activation of the cloned hIK channel by EBIO and show that the compound is obligatory Ca^{2+}-dependent. However, EBIO strongly increases channel activity at concentrations close to the resting intracellular levels of Ca^{2+}.

2. Materials and methods

2.1. Electrophysiology

The patch-clamp amplifier used was the EPC-9 (HEKA Electronics, Lambrect, Germany) controlled by computer via an ITC-16 interface. Data were acquired at 3–5 times the filtration rate using the Pulse software (HEKA-Electronics, Lambrect, Germany). Pipettes were pulled by a DMZ-Universal Puller (Zeitz Instrumente, Augsburg, Germany) from borosilicate glass (Modulohm, Copenhagen, Denmark). For single-channel experiments, the pipettes were coated with Sylgard and fire-polished to final resistances in the range 1.5–3.0 MΩ. A custom-made perfusion chamber (volume 15 μl) with a fixed AgCl–Ag-pellet electrode (In Vivo Metric, Healdsburg, CA), was mounted on the stage of an inverted microscope equipped with Nomarski interference contrasts.

2.2. Cells

Human embryonic kidney (HEK 293) cells stably transfected with the cDNA encoding placental hIK were used in all experiments (cloning and basic characterisation, see [5]). Cells were grown in DMEM-media supplemented with 10% FCS and 1% gentamycin and were passaged twice a week. Experiments reported in this paper were performed on passages 20–30.

2.3. Salt solutions

Extracellular: (1) standard Na^+ solution, 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES (pH 7.4); (2) high-K^+ solution, 144 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES (pH 7.4). Intracellular: (3) 144 mM KCl, 10 mM EGTA, 0–7.60 mM $CaCl_2$, $MgCl_2$ (1.20–5.60 mM) and 10 mM HEPES (pH 7.2). The free Ca^{2+} concentrations, varying between approximately 50 pM (hereafter called 0 Ca^{2+}) and 300 nM as well as the free Mg^{2+} concentration (constant = 1.00 mM in all solutions) were calculated with Eqcal-software (BioSoft, Cambridge, UK). EBIO was purchased from Sigma. Concentrated stock solutions were prepared in DMSO and diluted directly in the

relevant experimental solution. The final DMSO concentration used was always below 0.1%, which caused no interference with currents or membrane potentials. All chemicals were of the purest grade commercially available.

2.4. Experimental procedure

Coverslips containing the transfected HEK 293 cells were placed in the perfusion chamber and continuously superfused at a rate of 1 ml/min with extracellular salt solution. In the whole-cell voltage-clamp experiments, the series resistance as well as the cell capacitance were followed on-line. Initial series resistances (R_S) were below 5 MΩ and usually remained constant throughout the experiments. Experiments were invariably discarded if R_S increased above 10 MΩ or if slow capacitance cancellation failed. All experiments were performed at room temperature (21–25°C).

3. Results

3.1. Basic experiments

The experimental protocols used for recording of whole-cell currents from HEK 293 cells stably transfected with the hIK are outlined in Figs. 1 and 2. In the voltage-clamp experiments (Fig. 1), the cells were always held at a membrane potential of 0 mV, and currents were elicited by short potential steps or ramps repeated every 5 s. Fig. 1A shows typical ramp current responses from a cell exposed to symmetric K^+ solutions, and with increasing concentrations of free Ca^{2+} in the pipette. The expressed channels concomitantly changed the cell input resistance (from GΩ to hundreds of MΩ) as well as the whole-cell *I–V* characteristic (from linear or slightly superlinear to inward rectifying in symmetric K^+ solutions). In Fig. 1B the currents obtained at +80 mV has been plotted vs. the time after break-through to the whole-cell configuration. At 0 Ca^{2+} only a small leak current was observed even after long time of pipette/cell dialysis. However, at the higher Ca^{2+} concentrations (30, 100, and 300 nM), the current increased and saturated at distinct levels reflecting the equilibrium activation of the channels at each Ca^{2+} concentration. In all of the experiments described in the following, the pipette solution was allowed to equilibrate with the cell interior until a stable base-line was established (at least 3 min).

An alternative to the activation of native intermediate-conductance Ca^{2+}-activated K^+ channels

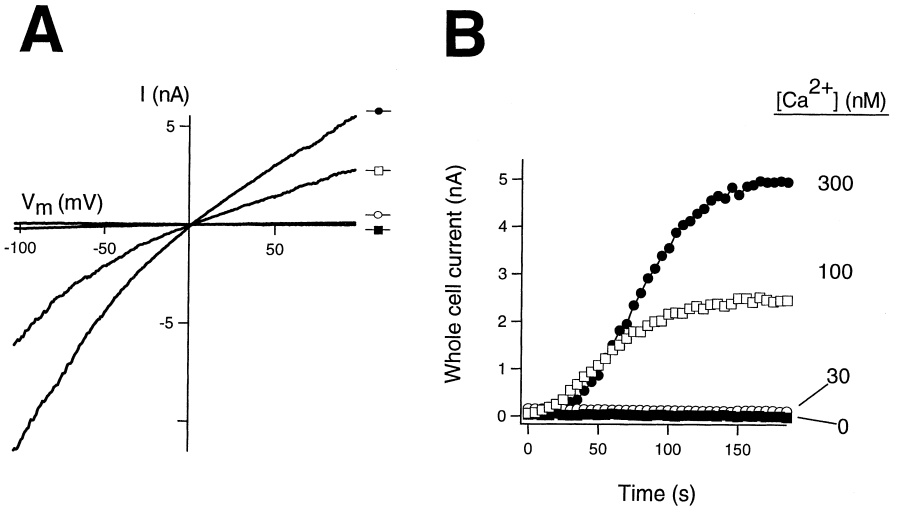

Fig. 1. HEK 293 cells stably transfected with hIK. Activation by Ca^{2+}. (A) Whole-cell currents obtained with symmetric K^+ solutions in response to voltage ramps. The traces are from different cells and were obtained after equilibration of the cytosol with pipette solutions containing the following buffered concentrations of free Ca^{2+}: ■, nominally Ca^{2+}-free (approximately 50 pM); ○, 30 nM; □, 100 nM, and ●, 300 nM. (B) Currents measured at 80 mV and plotted vs. the time after break-through to the whole-cell configuration. *I–V* ramps (±100 mV, 1 mV/ms) were elicited at a frequency of 0.2 Hz. Holding potential: 0 mV.

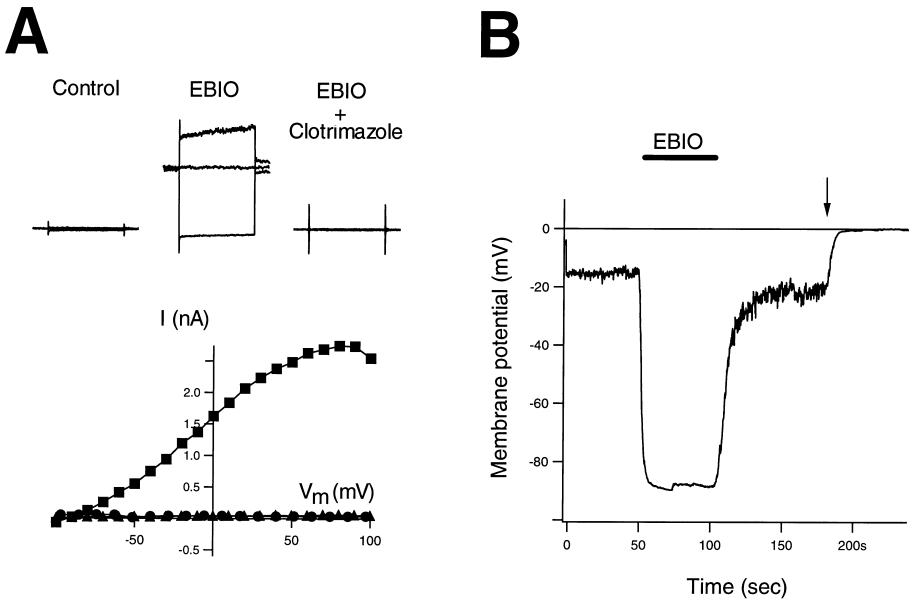

Fig. 2. HEK 293 cells stably transfected with hIK. Activation by EBIO. (A) Whole-cell voltage-clamp experiment with asymmetric Na^+/K^+ gradients (externally, solution 1; internally, solution 3 buffered at 65 nM free Ca^{2+}). Upper panels: current traces obtained by stepping from a holding potential of 0 to −100, 0, and 100 mV, respectively. The traces in the middle panel were obtained after superfusion with 100 μM EBIO. In the panel to the right, 1 μM clotrimazole was added to the EBIO-containing solution. Lower panel: current amplitudes plotted vs. membrane potentials in the range of −100 to 100 mV (duration, 100 ms; stimulation frequency, 0.2 Hz). ▲, Control; ■, after application of EBIO; ●, after co-application of EBIO and clotrimazole. (B) Whole-cell current-clamp experiment with asymmetric Na^+/K^+ gradients (as in A). EBIO was present in the bath solution during the time indicated by the bar. The arrow indicates the shift of extracellular solution to a solution where Na^+ was exchanged with a corresponding concentration of K^+ ($E_K = 0$ mV).

by raising intracellular Ca^{2+} is to apply the compound EBIO [4]. EBIO also activated the cloned hIK channel, and in symmetric K^+ solutions the compound quickly and reversibly potentiated an inward rectifying current which was similar to the current activated by Ca^{2+} [5].

In Fig. 2 the effect of EBIO in experiments with near physiological extracellular concentrations of Na^+ and K^+ has been illustrated. In all single-dose experiments an EBIO concentration of 100 μM was chosen, which is close to the previously estimated EC_{50} value for activation of hIK [5]. Fig. 2A shows a typical voltage-clamp study from a cell dialysed against a pipette solution with 65 nM Ca^{2+}. The upper panel shows the current traces obtained by stepping from a holding potential of 0 to −100 and 100 mV, respectively. The control currents were small (< 100 pA) and reversed close to 0 mV (predominantly leak current) as expected from the low pipette Ca^{2+} concentration. However, upon application of EBIO the current at 0 mV increased to 1.7 nA. The EBIO-induced current was reduced to −0.2 nA by stepping to −100 mV and augmented to 2.5 nA at 100 mV. Co-application of EBIO and the hIK channel blocker clotrimazole completely abolished the current. To obtain the *I–V* curves, the membrane voltage was varied from −100 to 100 mV in steps of 10 mV (lower panel). The current saturated at large positive voltages and shifted direction from outward to inward at a voltage close to the theoretical equilibrium potential for K^+ (−95 mV). These results indicate that EBIO-stimulated current maintains the very high K^+/Na^+ selectivity earlier demonstrated for native intermediate-conductance, Ca^{2+}-activated K^+ channels from human erythrocytes [10] as well as for hIK channels [5]. The instantaneous current responses obtained in the presence of EBIO shows that the gating of the drug-modulated channel remains voltage-independent as does the gating of hIK channels activated only by Ca^{2+}.

Fig. 2B shows a comparable current-clamp study from another cell buffered with a pipette Ca^{2+} concentration of 65 nM. A relatively noisy, but stable, membrane potential of −18 mV was initially re-

corded. Application of EBIO immediately hyperpolarised the cell to a new stable level at −88 mV characterised by a much lower noise content. Upon wash-out of EBIO the membrane potential repolarised towards the original level. The membrane potential noise observed at potentials positive to the equilibrium potential for K^+ directly reflects the membrane charging/discharging effects of opening and closing individual hIK channels. Accordingly, the noise also disappeared after superfusion with the high K^+ solution which make $E_K = 0$.

Control recordings with naive HEK 293 cells showed that neither high intracellular Ca^{2+} nor superfusion with EBIO activated hyperpolarising currents (not shown).

3.2. Ca^{2+}-dependency of EBIO modulation

To test the efficacy of EBIO as an hIK activator under controlled levels of channel activation, we studied the compound at various levels of intracellular free Ca^{2+} as set by the concentrations in the Ca^{2+}-buffered pipette solutions. Fig. 3A shows typical ramp current traces obtained before and after application of a fixed concentration of EBIO (100 μM) at 0, 30 and 300 nM intracellular Ca^{2+}, respectively. Note, that the scalings of the current axes varies strongly in order to emphasise the effect of EBIO at the different Ca^{2+} concentrations. In the experiment with 0 Ca^{2+}, no basic hIK channel activity was observed as judged from the very small (35 pA at 80 mV) and essentially linear *I–V* relation, which basically reflects the *I–V* relation of the naive HEK 293 cell clone used for transfection. Application of EBIO under these conditions neither influenced the size of the current nor the shape of the *I–V* curve. At 30 nM Ca^{2+}, a significant current was recorded (0.1 nA at 80 mV), and the weak inward rectification characterising hIK was evident. At this Ca^{2+} concentration, EBIO strongly activated the

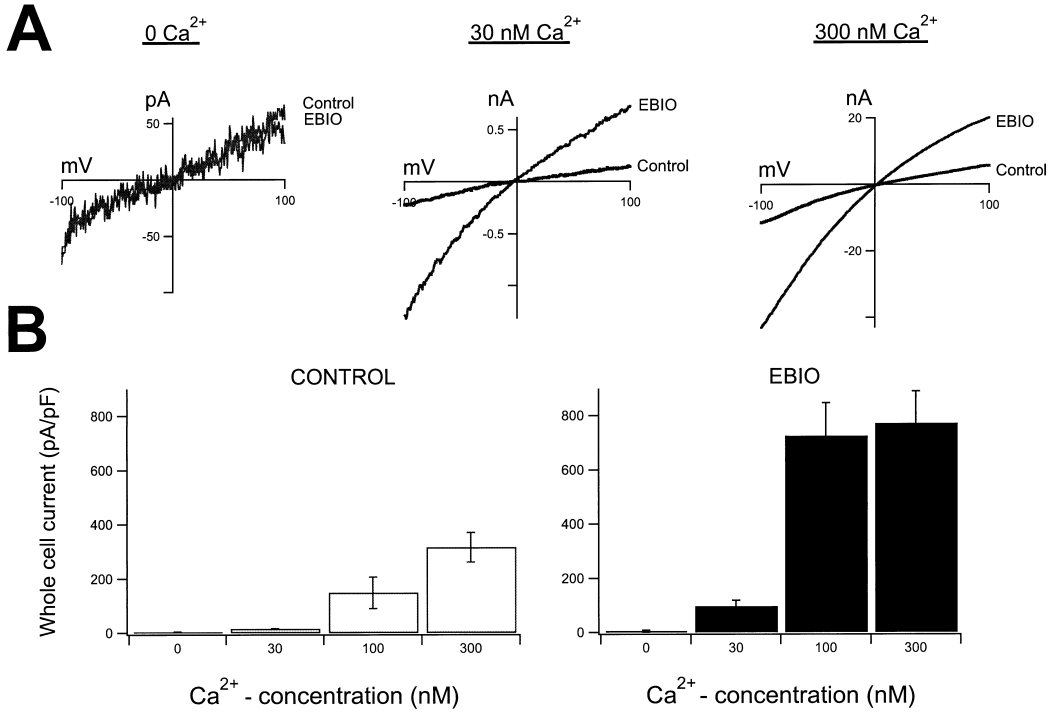

Fig. 3. Ca^{2+}-dependency of EBIO modulation of hIK. (A) Effect of 100 μM EBIO on ramp currents obtained after equilibration with pipette solutions (solution 3) buffered at 0, 30, and 300 nM free Ca^{2+}, respectively. The pipette solutions were allowed to equilibrate at least 3 min before application of EBIO. Note, that the current traces have been normalised to clarify the relative effects of the compound at the different Ca^{2+} concentrations. Stimulation protocols as described in legend to Fig. 1. (B) Histograms summarising the levels of stationary whole-cell currents (measured at 80 mV) obtained at the various concentrations of buffered intracellular free Ca^{2+} in the range of 0–300 nM before (left panel) and after EBIO application (right panel). All currents were normalised with respect to cell size (pA/pF). Error bars represents S.E.M.; *n* is between 6 and 8 for each Ca^{2+} concentration.

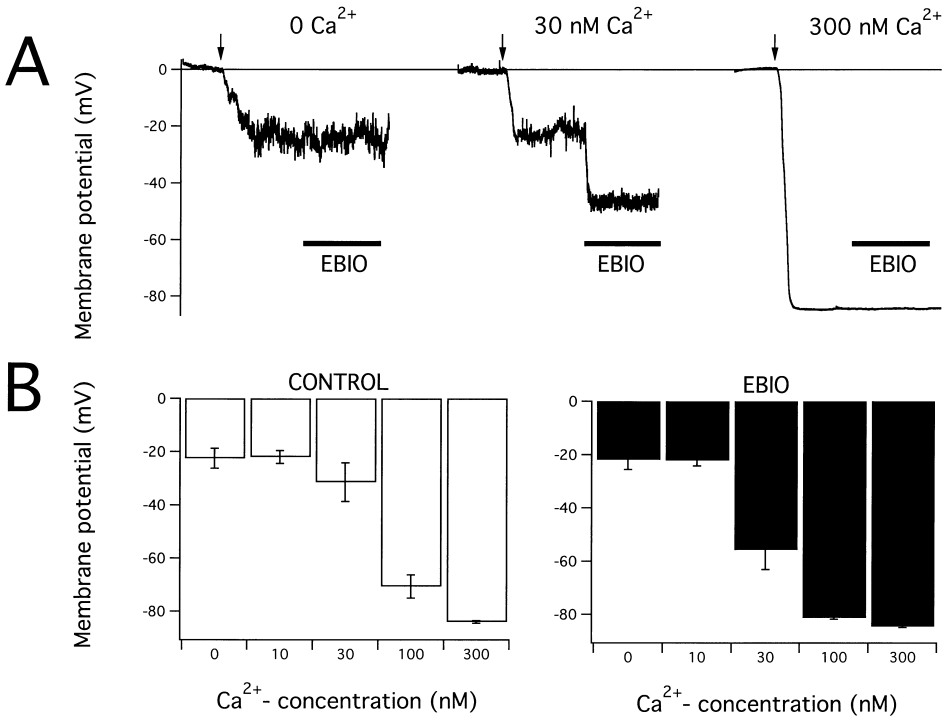

Fig. 4. Ca^{2+} and EBIO induced hyperpolarisations of hIK-transfected HEK 293 cells. (A) Recording of the membrane potentials from cells dialysed with pipette solutions (solution 3) buffered at 0, 30, and 300 nM Ca^{2+}, respectively. The cells were initially superfused with the high extracellular K^+ solution (solution 2) in which the electrodes were zeroed. After 2 min of recording (arrows), the high K^+ solution was exchanged with the standard extracellular Na^+ solution (solution 1). After 4 min, the cells were superfused with extracellular Na^+ solution containing 100 μM EBIO (indicated by the bars). (B) Summary of membrane potentials recorded after equilibration with pipette solutions containing 0, 10, 30, 100, and 300 nM Ca^{2+}, respectively. The stationary membrane potentials was recorded in the absence (left panel) and presence of 100 μM EBIO (right panel). Error bars represents S.E.M.; *n* is between 6 and 9 for each Ca^{2+} concentration.

current (to 0.6 nA at 80 mV). At 300 nM Ca^{2+}, the basic level of hIK activation was high and gave rise to an outward current of 3 nA at 80 mV. Even at this level of activation, EBIO application more than doubles the hIK specific current. In Fig. 3B, the mean whole-cell currents normalised with respect to cell size (pA/pF) has been plotted as a function of the intracellular Ca^{2+} concentration in absence (left) and in presence of EBIO (right). The basic hIK current increased as a function of intracellular Ca^{2+} with an apparent threshold for activation below 30 nM, and with no tendency to saturation even at 300 nM intracellular Ca^{2+}. Attempts to obtain the full dose–response curve for Ca^{2+} activation by equilibration with higher Ca^{2+} concentrations was generally unsuccessful, mainly due to problems with increasing series resistances during experiments. In presence of EBIO, the hIK currents were strongly increased at all concentrations of Ca^{2+} above 30 nM, and in contrast to the control experiments, the Ca^{2+} dose–response relation approached saturation already at 100 nM Ca^{2+}. However, there was no effect of EBIO at 0 Ca^{2+}, indicating that the ability of EBIO to stimulate hIK currents in the whole-cell configuration is strongly Ca^{2+}-dependent.

The interactions of EBIO and Ca^{2+} on hIK was quantified by current-clamp recordings with asymmetric ion gradients of Na^+ and K^+. Fig. 4A shows examples of such recordings with 0, 30 and 300 nM pipette Ca^{2+}. Giga-sealing and establishment of the whole-cell configuration was done in the high K^+ extracellular solution, and the initial membrane potential read-out was therefore close to zero in all experiments. Upon changing to the standard extracellular Na^+ solution, a negative basic membrane potential quickly developed. At 0 and 30 nM Ca^{2+} almost identical potentials around −20 mV developed, whereas the cell equilibrated with 300 nM hyperpo-

larised to −87 mV. No hyperpolarising effect of EBIO was observed at 0 Ca^{2+}, whereas the cell equilibrated with 30 nM Ca^{2+} responded to EBIO with an additional hyperpolarisation to −48 mV. In the cell equilibrated with 300 nM Ca^{2+}, the basic membrane potential was close to the equilibrium potential for potassium and, therefore, no additional hyperpolarisation was obtained with EBIO. Fig. 4B summarises the stable membrane potentials obtained at the various intracellular Ca^{2+} concentrations in the absence (left) and presence (right) of EBIO. In both cases, the membrane potentials raised steeply from limiting potentials of ≈−20 mV (−18.4±2.8 vs. −19.4±2.5 mV) at 0 Ca^{2+} to more than −80 mV (−84.0±0.5 vs. −84.6±0.3 mV) at 300 nM Ca^{2+}. The overall effect of EBIO was to leftward shift the membrane potential vs. intracellular Ca^{2+} curve, (the half maximal hyperpolarisation value was below 30 nM Ca^{2+} in presence of 100 μM EBIO). However, no hyperpolarising effect of EBIO was ever observed below 10 nM Ca^{2+}, indicating that the compound mediates Ca^{2+}-dependent hyperpolarisation of hIK-transfected HEK 293 cells.

High EBIO concentrations might trigger the hIK channels to open and hyperpolarise the cell membrane even in the virtual absence of Ca^{2}. Fig. 5A shows typical current-clamp recordings with cells equilibrated with 30 nM pipette Ca^{2+} (left) and 0 Ca^{2+} (right), respectively. With 30 nM Ca^{2+} a basic membrane potential of −25 mV was recorded. Superfusion with increasing EBIO concentrations in the range of 10 μM to 1 mM gradually hyperpolarised the cell. A significant hyperpolarisation occurred even at 10 μM (to −38 mV) and the maximal effect (to −83 mV) was attained at 300 μM. Remarkably, the wash-out of EBIO was fast and complete even after exposure to the highest concentration of 1 mM. Note, that the membrane potential oscillations present in this experiment reflect local concentration changes of EBIO in the experimental chamber during the solution shift. In the experiment with 0 Ca^{2+}, the basic membrane potential was −11 mV and there

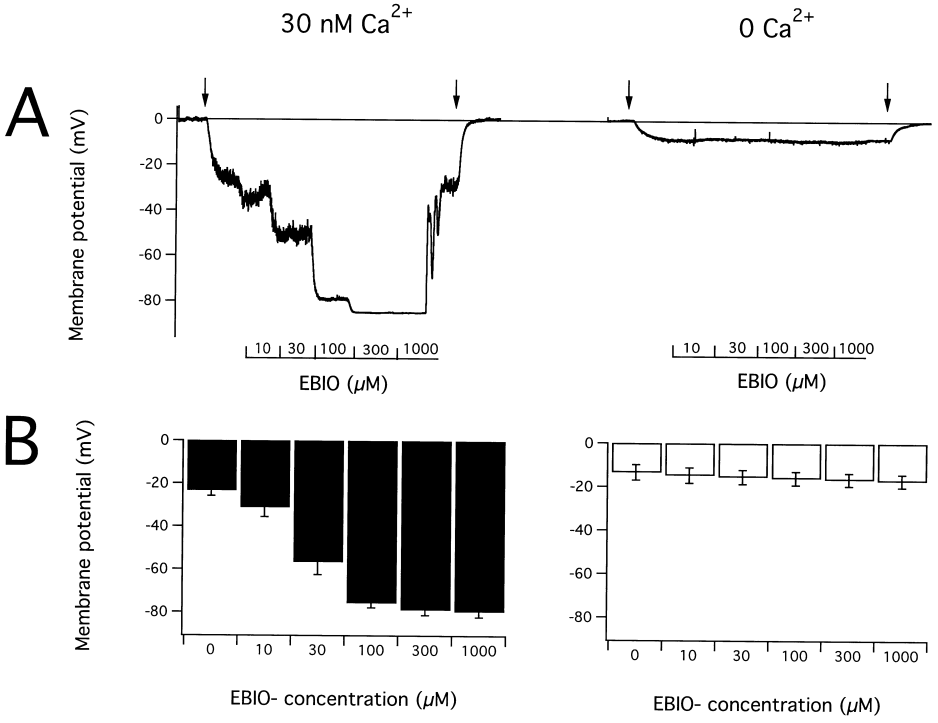

Fig. 5. EBIO dose–response experiments. (A) Current-clamp recordings showing the effect of increasing concentrations of EBIO on hIK-transfected cells. The pipette solutions were buffered at 30 (right panel) and 0 nM (left panel) Ca^{2+}, respectively. The arrows indicate shifts from the high K^{+} extracellular solution (solution 2) to the standard extracellular Na^{+} solution (solution 1) and back again. (B) Summary of membrane potential recordings with increasing concentrations of EBIO in a series of cells buffered at 30 nM Ca^{2+} ($n=6$) and a series of cells buffered at 0 Ca^{2+} ($n=6$). Experimental details as outlined in A.

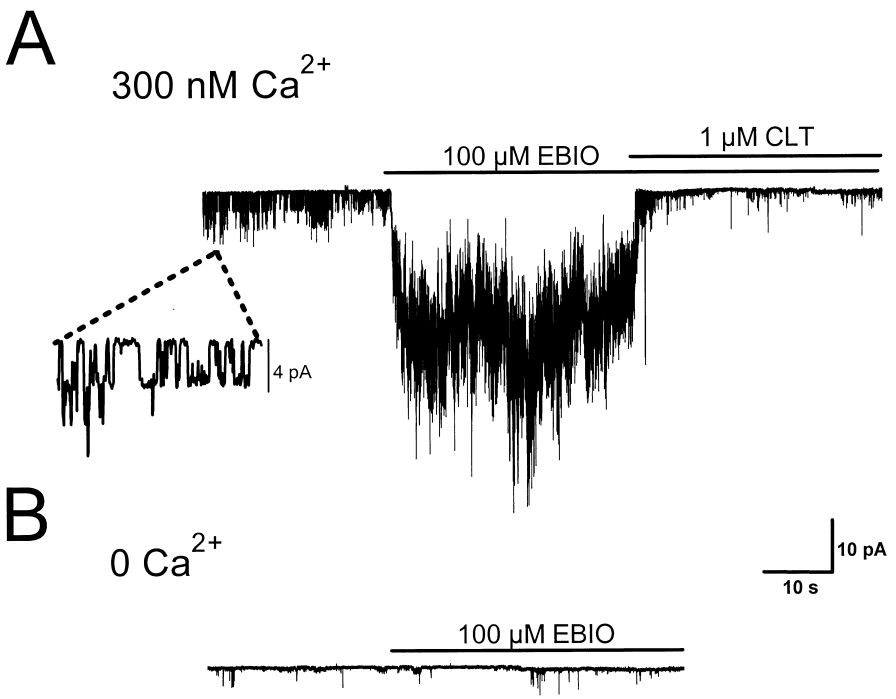

Fig. 6. EBIO modulation of single hIK channels. (A) A multichannel recording from an inside-out patch clamped at −100 mV. The patch was exposed to symmetrical K^+ solutions (solution 3 buffered at 300 nM Ca^{2+}). The expanded trace represents 1 s of recording and reveals up to three distinct single-channel current levels of −3.8 pA each. EBIO (100 μM) was applied to the bath after 30 s (lower bar) and clotrimazole (1 μM) was applied together with EBIO after 60 s. (B) No effect of EBIO applied to an inside-out patch superfused with solution 3 buffered at 0 Ca^{2+}. All traces were filtered at 300 Hz and acquired at a sample rate of 1000 points/s.

was no effect of superfusion with increasing concentrations of EBIO. Fig. 5B summarises the results from these series of experiments. At 30 nM internal Ca^{2+}, a mean basic membrane potential of -23.5 ± 2.4 mV and a maximally EBIO (1 mM) stimulated membrane potential of -79.7 ± 2.5 mV were found. At 0 Ca^{2+} the basic membrane potential was -13.5 ± 3.6 mV and the membrane potentials recorded in presence of 1 mM EBIO -17.4 ± 2.9 mV, indicating no significant activation of the channels.

3.3. Single-channel recordings

To demonstrate that EBIO acts directly on the channel or a closely associated accessory protein rather than via a receptor/second-messenger system, we performed single-channel recordings using excised inside-out patches. Fig. 6 shows the effect of EBIO on single-channels from inside-out patches exposed to 0 or 300 nM Ca^{2+} at the inside solution. Distinct single-channel gating events from at least three channels were observed in the patch exposed to 300 nM Ca^{2+}. Upon superfusion with EBIO (100 μM), the patch currents dramatically increased to a noisy current demonstrating the presence of multiple channels in the patch. Simultaneous application of EBIO and clotrimazole (1 μM) completely eliminated the noisy current, indicating that the EBIO-stimulated hIK channels maintain their basic sensitivity to clotrimazole. In the experiment with 0 Ca^{2+} (Fig. 6B), very few openings occurred (most likely representing gating events from endogenous HEK 293 channels) and neither the frequency nor the duration of these events were affected by superfusion with EBIO.

4. Discussion

The conditions for activation of the cloned hIK by EBIO was studied using HEK 293 cells stably expressing hIK channels.

Intracellular concentrations of free Ca^{2+} were varied in the range from 0 to 300 nM to study the

efficacy of EBIO at different basic levels of channel activation, and especially to elucidate whether channel activation might be induced by the compound itself or only in concert with Ca^{2+}. Basic hIK characteristic currents (inward-rectifying, time-independent, K^+-selective, clotrimazole-sensitive) developed gradually towards stable levels after break-through to the whole-cell mode. The plateau currents increased from 3 ± 1.6 to 315 ± 54.5 pA/pF (measured at 80 mV) without showing saturation, even at 300 nM Ca^{2+}. However, in presence of 100 μM EBIO the hIK current vs. Ca^{2+}-activation curve shifted towards lower Ca^{2+} concentrations and saturation was attained already at 100 nM Ca^{2+}. No EBIO-stimulated current was observed at 0 Ca^{2+}. The membrane potentials recorded in near-physiological transmembrane ion gradients were steeply Ca^{2+}-dependent, varying between −15 and −20 mV in the limit of low Ca^{2+} concentrations and −80 to −85 mV at 300 nM, indicating a dominating Ca^{2+}-dependent K^+ channel contribution to the total membrane conductance. EBIO at 100 μM strongly hyperpolarised cells at Ca^{2+}-concentrations between 30 and 100 nM. At 300 nM Ca^{2+}, where the basic membrane potential was already close to the equilibrium potential for K^+, EBIO could not hyperpolarise the cells any further. At 0 Ca^{2+}, EBIO-induced hyperpolarisations were even not obtainable at EBIO concentrations as high as 1 mM. In contrast, at 30 nM Ca^{2+} EBIO hyperpolarised the membrane in the low μM concentration range. Therefore, the overall action of EBIO on the cloned channel is not to act as a gating molecule per se. In whole cells, EBIO effectively induces a left-shifting of the Ca^{2+}-activation curve. It remains to be settled if this effect, at the molecular level, represents a modification of the Ca^{2+} binding properties of hIK. In this respect, it is noteworthy that Ca^{2+} binding is different in nature among the three classes of Ca^{2+}-activated K^+ channels. The BK channel α-subunit forms a genuine binding-site for Ca^{2+} [11] and is perfectly capable of performing Ca^{2+}-dependent gating, whereas the SK α-subunits have no such site and combine constitutively with calmodulin to obtain its Ca^{2+}-dependency [12]. A recently published paper [13] indicates that the gating of hIK also depends on Ca^{2+} binding to tightly bound calmodulin. Therefore, it cannot be excluded that EBIO improves calmodulin Ca^{2+} binding and/or the calmodulin–channel interaction.

EBIO was originally studied for its effects on the short circuit currents through cultured T-84 epithelia, a model for colonic NaCl/water secretion, and the effects were confirmed in intact murine colonic epithelium [14]. The EBIO-stimulated secretion was blocked dose-dependently by charybdotoxin as well as by clotrimazole [8]. Patch-clamp studies clearly showed that the effect of the compound was to increase the activity of the basolateral, inward rectifying, intermediate-conductance, Ca^{2+}-activated K^+ channel, thus resembling the effect of Ca^{2+} mobilising secretagogues, like carbachol and taurodeoxycholate [15]. However, the stimulatory efficacy of EBIO was found to be superior to raising intracellular Ca^{2+} [4]. Therefore, it was suggested that the compound acted by increasing in parallel the luminal cAMP-dependent Cl^- conductance as well as the basolateral Ca^{2+}-dependent K^+ conductance, thus enabling an enhanced and prolonged secretory response. EBIO increased the open state probability of the K^+ channels in inside-out patches, indicating that the effect was exerted via a direct action on the channel protein rather than via a secondary mechanism involving Ca^{2+} or another second messenger molecule. The EBIO stimulation observed in inside-out patches was also shown to be Ca^{2+}-dependent, since the effect disappeared by lowering the Ca^{2+} concentration from 200 nM to zero [14].

Despite the clear Ca^{2+}-dependence of the EBIO effect, it is obvious that EBIO is effective at Ca^{2+} concentrations close to or even below the resting level of intracellular Ca^{2+}. At this concentration range of Ca^{2+}, the open-state probability of hIK channels is low [15]. This indicates that EBIO – or a compound with a similar mechanism of action – might be an extremely effective way of hyperpolarising cells expressing hIK, even without a concurrent cytosolic Ca^{2+} increase. The powerful activating effect of EBIO on the short-circuit current in otherwise unstimulated secretory epithelia, indicates that the basic intracellular Ca^{2+} concentration close to the basolateral membrane is sufficient to sustain EBIO activation of the Ca^{2+}-activated K^+ conductance.

The Ca^{2+} sensitivity of EBIO action on hIK differs qualitatively from the mechanism by which compounds, such as the benzimidazolone, NS1619 and

the substituted diphenylurea, NS1608, activates the BK channel subtype of Ca^{2+}-activated K^+ channels [16]. Activation of BK channels by these compounds was demonstrated to be a Ca^{2+}-independent process characterised by a dose-dependent leftward shift of the voltage activation curve. We found no activating effect of NS1608 and NS1619 on hIK at concentrations, which activate BK channels (not shown). Consistent with these findings, NS1619 has recently been shown to exert a blocking effect on the intermediate-conductance Ca^{2+}-activated K^+ channel in aortic endothelial cells [17] at concentrations above 10 μM. Furthermore, we did not find any effect of EBIO on the cloned BK channels (not shown).

The mechanistic similarity between the effects of EBIO on the cloned hIK and on the native IK channels strengthens the notion that the cloned hIK is identical to or closely related to the basolateral Ca^{2+}-activated K^+ channels of intermediate-conductance present in colonic epithelia. The cloned hIK may therefore constitute a relevant molecular target for the focused development of new drugs acting on this channel. hIK is almost exclusively expressed outside excitable tissues and predominantly in epithelia and blood cells [5,15,18]. Therefore, a therapeutic application for openers of this channel may be various secretory disorders, such as cystic fibrosis (decreased airway and intestinal secretion) and Sjøgrens syndrome (decreased saliva secretion).

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