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Tenidap, a novel anti-inflammatory agent, is an opener of the inwardly rectifying K^+ channel hKir2.3

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Abstract

We studied the effect of a novel anti-inflammatory agent, tenidap, on a cloned inwardly rectifying K^+ channel, hKir2.3. Tenidap (a) potently potentiated $^{86}Rb^+$ efflux through hKir2.3 channels expressed in Chinese hamster ovary cells ($EC_{50} = 402$ nM), (b) reversibly and dose-dependently increased whole-cell and macro-patch hKir2.3 currents (maximum whole-cell current response to tenidap was $230 \pm 27\%$ of control; $EC_{50} = 1.3$ μM.), and (c) caused dose-dependent and Ba^{2+}-sensitive membrane hyperpolarizations and concurrent decreases in input resistance. Potentiation of hKir2.3 by tenidap was unaffected by inhibitors of phospholipase A_2, protein kinase C, or arachidonic acid metabolic pathways. The action of tenidap was not intracellular. Tenidap also had little or no effect on currents flowing through hKir2.1, Kv1.5, and μ1 Na^+ channels. Our results demonstrate that tenidap is a potent opener of hKir2.3 and suggest that it can serve as a valuable pharmacological tool for studying physiological and pathological processes involving Kir2.3.

Keywords: K^+ channel, inwardly rectifying; Kir2.3; Tenidap; Arachidonic acid; Potentiation; Opener

1. Introduction

Inwardly rectifying potassium channels (Kir) are characterized by a subunit topology of two transmembrane domains surrounding a pore region and by the distinctive property of conducting current more readily in the inward direction (Kubo et al., 1993). Seven subfamilies of Kirs, Kir1.0–Kir7.0, have been cloned. In addition to primary sequence differences, channels from the Kir2.0 subfamily are functionally distinguished from those of the other subfamilies by their constitutive activity and strong inward rectification. Of particular interest in this subfamily is Kir2.3, which is highly expressed in the human brain and heart (Périer et al., 1994) and is thought to play a critical role in regulating cellular excitability (Hille, 1992). A number of signaling molecules have been identified to modulate this channel, including ATP (Collins et al., 1996), protein kinase C (Henry et al., 1996), G-protein βγ subunits (Cohen et al., 1996), Mg^{2+} (Chuang et al., 1997) and H^+ (Coulter et al., 1995; Zhu et al., 1999), all of which inhibit the channel. As with other Kirs, phosphatidylinositol 4,5-bisphosphate (PIP_2) appears to be essential for maintaining the channel activity (Zhang et al., 2001). Recently, we described the selective activation of hKir2.3 by several long-chain fatty acids, including arachidonic acid (Liu et al., 2001). In this study, we report the identification of an exogenous activator of hKir2.3, tenidap.

Tenidap is a novel investigational drug with demonstrated clinical efficacy for rheumatoid arthritis (Bondeson, 1996). One of the major effects of tenidap is its potent inhibition of cyclooxygenase (Moilanen et al., 1988). It has also been shown to inhibit arachidonic acid release and cytokine production in activated macrophages (Bondeson and Sundler, 1994), release Ca^{2+} from intracellular stores (Fujii et al., 1995), and lower intracellular pH (McNiff et al., 1994).

Effects of tenidap on ion channels have not been studied extensively. In one report, tenidap appears to inhibit Ca^{2+} influx through voltage-gated Ca^{2+} channels in mouse pituitary tumor cells (Cleveland et al., 1993). Another study

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shows that tenidap enhances the membrane permeability in mouse macrophages, apparently by increasing the affinity of extracellular ATP for binding to and activating $P2X_7$ receptor channels (Sanz et al., 1998). During a random screening effort, we identified tenidap as a potent activator of hKir2.3, which we report here using $^{86}Rb^+$ flux, voltage- and current-clamp assays. To our knowledge, this is the first report of an exogenous opener of Kir2.3. Our results suggest that tenidap may be a very useful tool for studying Kir2.3-related physiological and pathological processes, particularly those in the brain and heart.

2. Materials and methods

2.1. Channel expression and cell culture

Human Kir2.1 and Kir2.3 were stably expressed in a mutant line of Chinese hamster ovary (CHO) cells (Steglich and Scheffler, 1982) as previously described (Liu et al., 2001). Cells stably expressing hKir2.1 or hKir2.3 were grown in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum, non-essential amino acids, G-418 (400 μg/ml) and putrescine (500 μM) in a humidified, 37 °C incubator with 10% CO_2. For $^{86}Rb^+$ flux experiments, cells were plated at a density of 3.7×10^4 cells/well in 96-well plates 1 day before use. Human Kv1.5 was stably expressed in LM-(TK^-) cells grown in high glucose DMEM supplemented with 10% fetal bovine serum and G418 (250 μg/ml) at 37 °C with 10% CO_2. Rat skeletal muscle μ1 Na^+ channels were stably expressed in CHO cells grown in Ham's F-12 medium supplemented with 10% heat-inactivated fetal bovine serum, streptomycin (50 μg/ml), and penicillin (50 units/ml) at 37 °C with 5% CO_2.

2.2. $^{86}Rb^+$ flux assay

Cells were plated at a density of 3.7×10^4/well in a 96-well plate 1 day before use and were incubated for 4 h with $^{86}Rb^+$ (1 μCi/ml) in the culture medium before the start of experiment. They were subsequently rinsed four times to remove extracellular $^{86}Rb^+$ with a modified Earle's balanced salt solution (MEBSS) containing (mM) 132 NaCl, 1.8 $CaCl_2$, 5.4 KCl, 0.8 $MgCl_2$, 10 glucose, and 10 HEPES, pH = 7.4. Compounds were added to the cell-bathing solution at the start of experiment. After 50 min of flux, $^{86}Rb^+$ released from cells into the extracellular solution was collected. The cells were then lysed with 0.1% sodium dodecyl sulfate. The amounts of $^{86}Rb^+$ in both the extracellular solution and the cell lysates were measured with a Wallac 1450 MicroBeta Trilux liquid scintillation and luminescence counter (PerkinElmer Life Sciences, Turku, Finland). The total $^{86}Rb^+$ uptake after 4-h incubation was ~1500 counts/min. All experiments were conducted at room temperature (20–22 °C).

2.3. Electrophysiology

Standard whole-cell and patch-clamp techniques were used (Hamill et al., 1981). Recording pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) and fire polished. Solution-filled pipettes typically had a resistance of 1–2 MΩ. For whole cell recording, at least 85% of the series resistance (~2–5 MΩ) was compensated. Currents were amplified, digitized and filtered (5 kHz) with an AXOPATCH 200B patch-clamp amplifier and 1200 series DigiData digitizer (Axon Instruments, Foster City, CA). Current records of hKir2.1 and hKir2.3 were sampled at 2 kHz and leak subtracted from those in the presence of 3 mM Ba^{2+} (see figure legends for other details). For Kv1.5, cells were depolarized to +10 mV for 1.5 s from a holding potential of −80 mV. Currents were sampled at 1 kHz. The inter-pulse interval was 30 s. The duration of tenidap application was 5 min. A P/4 procedure was performed for leak current subtraction. In tonic recording of μ1 Na^+ channels, cells were depolarized to 0 mV for

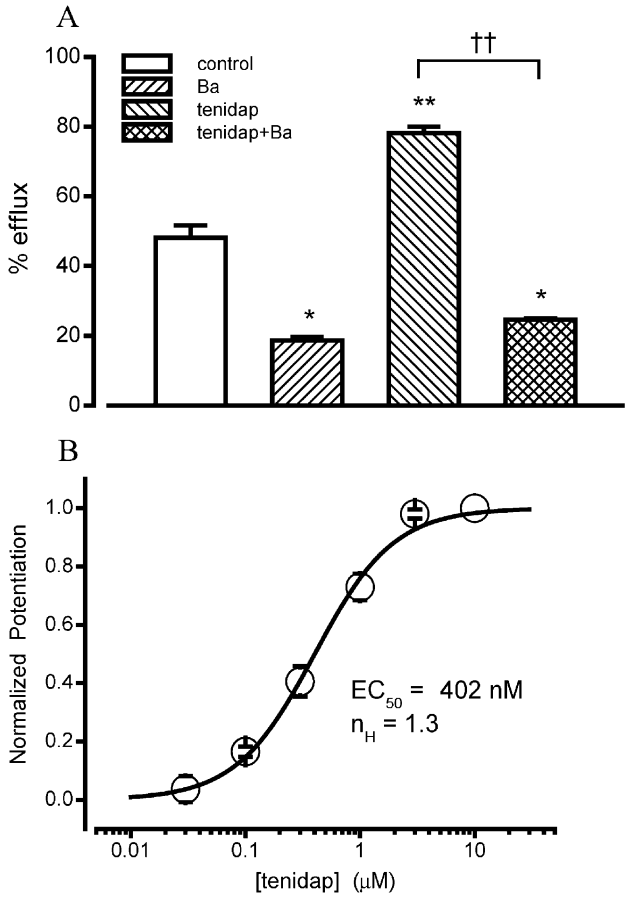

Fig. 1. Tenidap increased $^{86}Rb^+$ efflux through hKir2.3 channels. (A) Percentage efflux through CHO cells expressing hKir2.3 in control, 3 mM Ba^{2+}, 10 μM tenidap or 10 μM tenidap + 3 mM Ba^{2+}. Comparison between tenidap alone and tenidap + Ba^{2+} is indicated by a connector. (B) Dose-dependence of tenidap-induced $^{86}Rb^+$ efflux through hKir2.3 channels. Data ($n = 4$) were fitted to a logistic function (solid line).

165 ms from a holding potential of − 120 mV (the sampling rate was 100 kHz for the first 10 ms and 5 kHz thereafter). The inter-pulse interval was 60 s. This was followed by a phasic protocol in which, for 15 s, cells were depolarized once every 0.5 s to 0 mV for 20 ms from a holding potential of − 120 mV (sampling rate = 100 kHz). These protocols were run first in control and then in the tenidap-containing solution (tenidap was present for 5 min during the tonic protocol). All experiments were conducted at room temperature (20–22 °C).

2.4. Solutions and chemicals

Cells were perfused with MEBSS for all whole-cell recordings. For whole-cell recording of hKir2.1 and hKir2.3, the intracellular pipette solution contained (mM): 5 NaCl, 40 KCl, 100 KF, 5 EGTA, 3 EDTA, 10 HEPES, and 5 glucose, pH = 7.4. Outside-out patches were perfused with a solution substituting 70 mM KCl for an equal concentration of NaCl in MEBSS. The pipette solution was identical to that used in whole-cell recording. For whole-cell recording of Kv1.5, the pipette solution contained (mM): 40 KCl, 100 KF, 5 NaCl, 2 $MgCl_2$, 5 K-EGTA, 10 HEPES, and 5 Glucose, pH = 7.4. For whole-cell recording of μ1 Na^+ channels, the pipette solution contained (mM): 135 CsF, 10 CsCl, 5 NaCl, 5 Cs-EGTA, and 10 HEPES, pH = 7.4.

The stock solution of tenidap (10 mM in dimethyl sulfoxide (DMSO)) was stored at − 20 °C. The stock solutions of indomethacin, 5,8,11,14-eicosatetraynoic acid (ETYA), nordihydroguaiaretic acid (NDGA), 4-bromophenacyl bromide (4BPB), 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine·2HCl (H7) (all at 10 mM in DMSO) and 2-[1-(3-(Amidinothio)propyl)-1*H*-indol-3-yl]-3-(1-methylindol-3-yl)maleimide methanesulfonate (Ro 31-8220; 1 mM in DMSO) were stored in small aliquots at − 80 °C and were freshly thawed and diluted into MEBSS or pipette solution each time before use. Bovine serum albumin (10 μM) was directly dissolved in bath or pipette solution. Tenidap was identified during the course of screening of Abbott (Abbott Park, IL) libraries using the $^{86}Rb^+$ flux assay. ETYA and Ro 31-8220 were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). All other chemicals were purchased from Sigma (St. Louis, MO).

2.5. Data analysis

Data from voltage- and patch-clamp recordings of hKir2.1 and hKir2.3 were analyzed as previously described (Liu et al.,

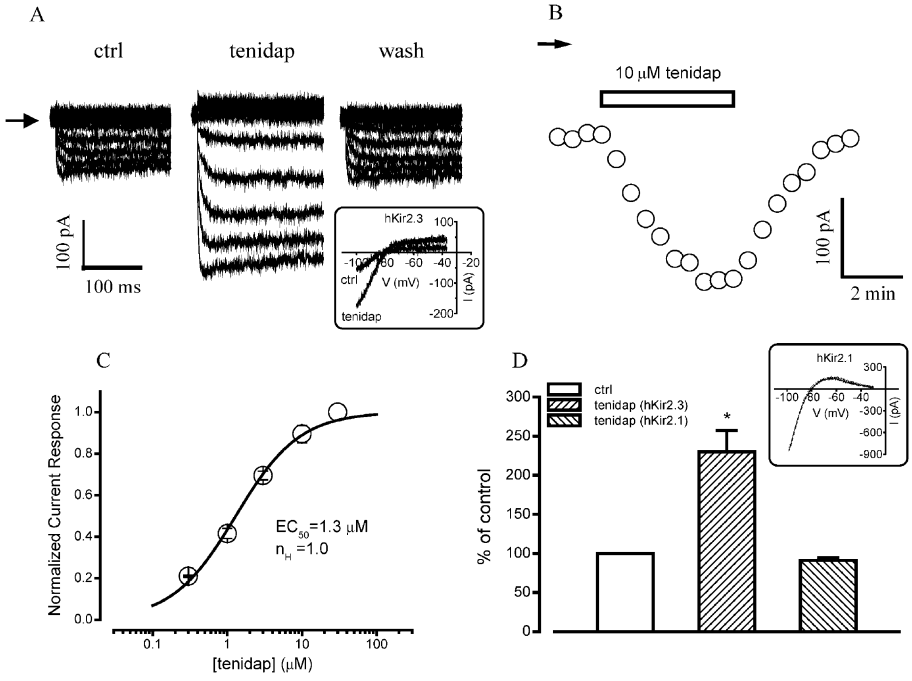

Fig. 2. Tenidap increased hKir2.3 but not hKir2.1 currents. (A) Whole-cell hKir2.3 currents in response to a series of 250 ms voltage steps from − 127 to + 23 mV (10 mV increments) before (ctrl), during (tenidap) and after (wash) application of 10 μM tenidap. Inter-pulse intervals were 3 s. Data were leak subtracted for each potential from corresponding traces in 3 mM Ba^{2+}. Arrow indicates zero current level. Holding potential was − 77 mV. Inset: whole-cell hKir2.3 *I–V* curves in control and 10 μM tenidap (both Ba^{2+}-subtracted) elicited by voltage ramps (− 100 to − 37 mV at 0.3 V/s). Reversal potential was − 80 mV for both the control and tenidap traces. (B) Time course of tenidap (10 μM) activation and wash (at − 97 mV). Arrow indicates zero current level. (C) Dose-dependence of potentiation of hKir2.3 whole-cell current by tenidap. Data from six cells were normalized individually to responses at 30 μM tenidap and fitted to a logistic function (solid line). (D) Effects of tenidap on whole-cell hKir2.1 (30 μM; $n = 3$) or hKir2.3 (10 μM; $n = 8$) currents. Inset: hKir2.1 whole-cell *I–V* curves (Ba^{2+} subtracted) elicited by a voltage ramp protocol (− 100 to − 30 mV at 0.3 V/s) in control and wash (solid lines) and 30 μM tenidap (dashed line). For (B), (C) and (D) (except the inset in D), cells were hyperpolarized to − 97 mV for 250 ms once every 30 s from a holding potential of − 77 mV. The average current amplitudes during the last 50 ms of the responses at − 97 mV were used in the calculations.

2001). For current-clamp recording of hKir2.3, hyperpolarizing current ranging from 0 to 50 pA was injected into each cell. The resulting membrane potential was plotted as a function of the injected current and the data were fitted to a linear function. The cell input resistance was obtained from the slope factor of the fit. $^{86}Rb^+$ efflux was calculated as the ratio (in %) of the amount of $^{86}Rb^+$ released into the extracellular solution and the total amount of $^{86}Rb^+$ contained in the extracellular solution and the cell lysates. The tenidap dose–response data from $^{86}Rb^+$ flux experiments were normalized for individual experiments to the difference between the efflux value in control (MEBSS) and that in 10 μM tenidap. They were fitted to a logistic function given by $r = 1 - 1/[1+(c/EC_{50})^{n}_{H}]$, where r is the normalized response, c is the tenidap concentration, EC_{50} is the concentration at which 50% of the maximum tenidap response is reached and n_H is the Hill coefficient. For Kv1.5, the average current amplitude over the last 50 ms of depolarization was used for calculations. For μ1 Na^+ channels, the average peak current amplitude of the last three traces in the phasic protocol was used. Data were expressed as mean ± S.E.M. Where appropriate, two-tailed Student's *t*-tests were performed to determine the statistical significance of compound effects. Statistical significance is denoted by *($p < 0.05$) and **($p < 0.01$) (compared to control values) or †($p < 0.05$) and ††($p < 0.01$) (for second comparisons). The junction potentials in the whole-cell experiments on hKir2.1 and hKir2.3 were corrected. All data were analyzed off-line using pCLAMP6 (Axon Instruments) and Microcal Origin (version 5.0, Microcal Software, Northampton, MA).

3. Results

3.1. Tenidap potently and dose-dependently increased $^{86}Rb^+$ efflux through hKir2.3 channels

Under control conditions, nearly 50% of the total amount of intracellular $^{86}Rb^+$ fluxed out of the CHO cells expressing hKir2.3 in 50 min (Fig. 1A). This efflux was largely inhibited by Ba^{2+} (3 mM), suggesting that it primarily occurred through hKir2.3 channels expressed in these cells. The residual efflux (<20%) was probably through non-specific pathways since CHO cells not expressing hKir2.3 also had a similar level of efflux (data not shown). Incubation with tenidap (10 μM) significantly enhanced the amount of efflux (0.1% DMSO, which was present in the tenidap solution, did not change efflux; data not shown). The total efflux in the presence of 10 μM tenidap was inhibited by Ba^{2+} (3 mM) to a level similar to that in the absence of

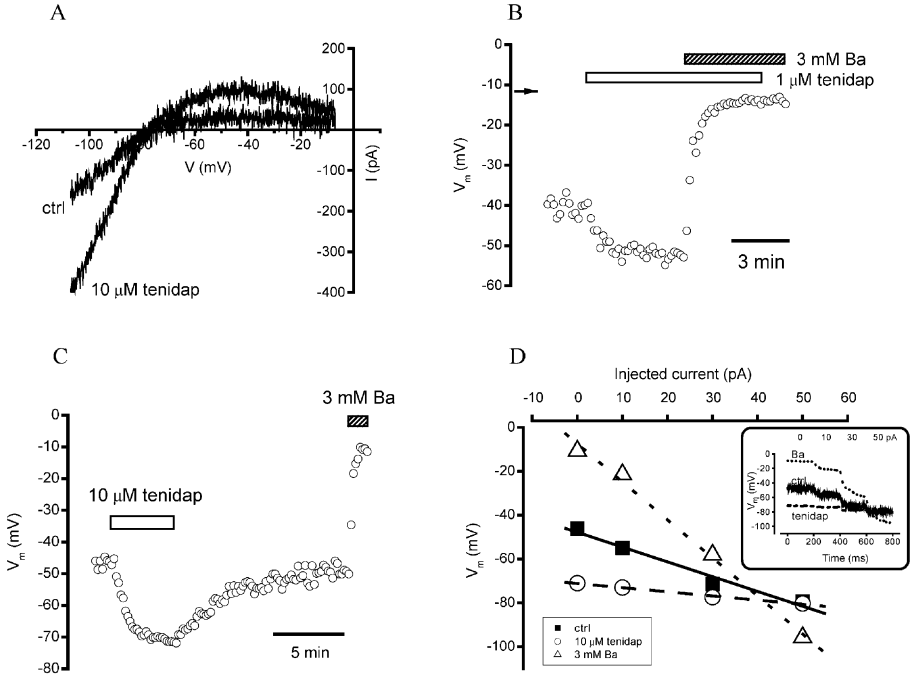

Fig. 3. Tenidap decreased input resistance and resulted in hyperpolarization in CHO cells expressing hKir2.3. (A) Whole-cell hKir2.3 current–voltage relationships in control and 10 μM tenidap. Currents (Ba^{2+} subtracted) were elicited by voltage ramps from −108 to −8 mV (0.3 V/s). (B) Tenidap (1 μM) caused a negative shift in the resting membrane potential. Co-application of Ba^{2+} (3 mM) with 1 μM tenidap depolarized the cell above the control level and to the same extent as Ba^{2+} (3 mM) alone did. Arrow represents V_m in 3 mM Ba^{2+} at the start of the experiment. (C) Hyperpolarization induced by 10 μM tenidap was reversible and larger than that induced by 1 μM tenidap. No current injection in (B) or (C). (D) Membrane potential as a function of injected current in control, tenidap (10 μM) or Ba^{2+} (3 mM). Cell was clamped at each current level for 200 ms in succession in the following sequence: 0, −10, −30 and −50 pA (inset). The average membrane potential amplitude during the last 20 ms of the response to each current injection was used in the calculations. Straight lines were linear fits to the data. The cell input resistance values obtained from the fits were: 674 MΩ (control), 189 MΩ (10 μM tenidap) and 1735 MΩ (3 mM Ba^{2+}), respectively. Data in (A), (B), (C), and (D) are from a single cell.

tenidap, indicating that the tenidap-induced efflux was also Ba^{2+}-sensitive and therefore likely through hKir2.3 channels. Tenidap enhanced $^{86}Rb^{+}$ efflux potently and dose-dependently with an EC_{50} value of 402 nM (Fig. 1B).

3.2. Tenidap potently, reversibly and selectively increased hKir2.3 currents

In Fig. 2A, a series of voltage steps (− 127 to + 23 mV) elicited whole-cell, Ba^{2+}-sensitive hKir2.3 currents that were strongly inwardly rectifying. Bath application of tenidap (10 μM) significantly and reversibly increased the current amplitude throughout this voltage range. The tenidap-induced current remained inwardly rectifying and had the same reversal potential as the control current (Fig. 2A, inset). These results, along with those in Fig. 1, are consistent with an effect of tenidap on hKir2.3 rather than through non-specific pathways. The effect of tenidap was fast and could be observed within seconds of drug application. Recovery from the tenidap action was similarly fast (Fig. 2B). The current potentiation by tenidap was concentration dependent with an EC_{50} value of 1.3 μM (Fig. 2C). At − 97 mV, the average current amplitude in 10 μM tenidap was 230 ± 27% of that in control (Fig. 2D). Tendiap also reversibly increased hKir2.3 currents in outside-out patches (Fig. 4B). Interestingly, even relatively high concentrations (e.g., 30 μM) of tenidap failed to affect currents flowing through hKir2.1 channels, which shares ~60% amino acid identity with hKir2.3 (Fig. 2D). In addition, tenidap (30 μM) had little or no effect on hKv1.5 and rat μ1 Na^{+} channels [70.8 ± 4.0% (n = 3) and 92.3 ± 5.8% (n = 3) of control, respectively].

3.3. Tenidap caused membrane hyperpolarization in CHO cells expressing hKir2.3

Because the value of the resting membrane potential in CHO cells expressing hKir2.3 results from a balance between the hyperpolarizing hKir2.3 current and other currents, including leak currents, it may be possible to hyperpolarize the cells by increasing hKir2.3 activity. We tested this idea by examining the ability of tenidap to lower the cell membrane potential in current-clamp experiments. Fig. 3 shows an example of such an experiment. In this cell, although the reversal potential for the hKir2.3 current was around − 80 mV (Fig. 3A), the initial resting membrane potential was near − 40 mV (Fig. 3B,C). Tenidap (10 μM) enhanced both inward and outward hKir2.3 currents in the cell (Fig. 3A), similar to the results in Fig. 2A. It also reversibly and dose-dependently hyperpolarized the cell by 13 and 25 mV at 1 and 10 μM, respectively (Fig. 3B,C). [In two other cells with more negative initial resting membrane potentials, 10 μM tenidap caused smaller, but significant hyperpolarizations (7 and 9 mV, respectively).] Ba^{2+} (3 mM) not only reversed this hyperpolarization, but also depolarized the cell further, to the extent to which Ba^{2+} alone depolarized the cell (Fig. 3B). This indicates, as would be expected from results in Fig. 1, that Ba^{2+} blocked both

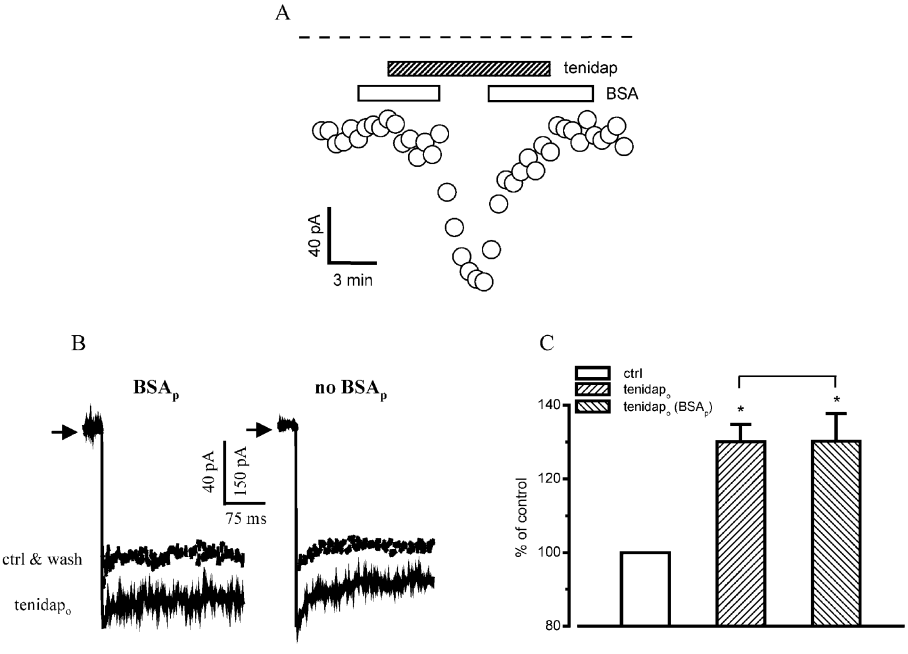

Fig. 4. Tenidap did not appear to act from an intracellular site. (A) Effects of bovine serum albumin on potentiation of whole-cell hKir2.3 current by tenidap (10 μM). Dashed line represents zero current level. Same voltage protocol as in Fig. 2B. (B) Representative, leak-subtracted current traces from two outside-out macro-patches. Extracellular tenidap (tenidap$_o$; 30 μM) was perfused onto the patches with (left panel) or without (right panel) bovine serum albumin (10 μM) in the pipette (BSA$_p$). Arrows indicate zero current level. Patches were held at 0 mV and hyperpolarized to − 120 mV for 250 ms. (C) Average responses to extracellular application of 30 μM tenidap to outside-out patches with (n = 4) or without (n = 3) bovine serum albumin (10 μM) in the pipette.

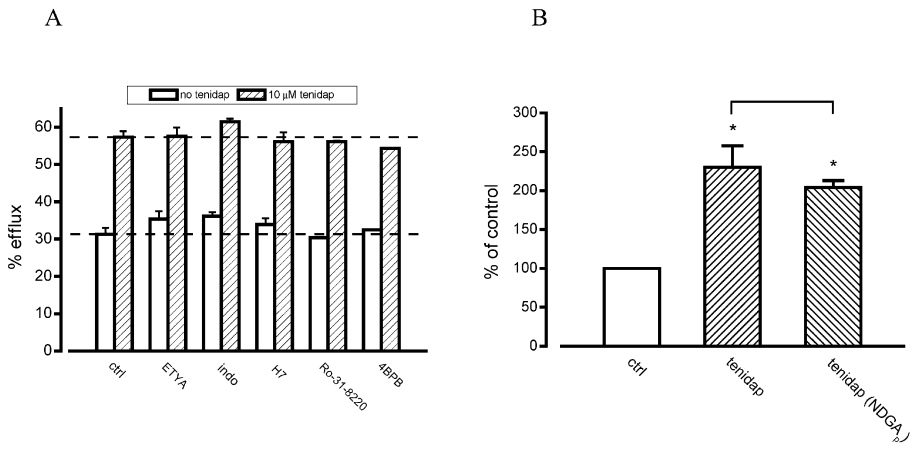

Fig. 5. Effect of inhibitors of phospholipase A_2, protein kinase C and arachidonic acid metabolism on potentiation of hKir2.3 by tenidap. (A) $^{86}Rb^+$ efflux through hKir2.3 channels ($n=2$). Open bars: $^{86}Rb^+$ efflux in control solution or in the presence of an inhibitor alone (from left to right: control, 100 μM ETYA, 30 μM indomethacin, 30 μM H7, 3 μM Ro 31-8220, and 10 μM 4BPB). Hatch bars: same conditions as above + 10 μM tenidap. Non-specific efflux was excluded by subtracting the efflux in 3 mM Ba^{2+} from the total efflux. (B) Potentiation of whole-cell hKir2.3 current by 10 μM tenidap alone (from Fig. 2D) or 10 μM tenidap with 50 μM NDGA in the recording pipette (NDGA$_p$; $n=6$). Extracellular NDGA (50 μM) blocked the $^{86}Rb^+$ efflux and current through hKir2.3, but inclusion of NDGA (50 μM) in the pipette did not appear to affect hKir2.3 current (data not shown).

the constitutive and tenidap-induced hKir2.3 currents. At the same time at which the resting membrane potential in Fig. 3C was recorded, the input resistance of the cell was also monitored by injecting varying amounts of hyperpolarizing currents (Fig. 3D, inset). Consistent with tenidap opening hKir2.3, the cell input resistance was decreased by tenidap (10 μM) and increased by Ba^{2+} (3 mM) (674 MΩ in control, 189 MΩ in 10 μM tenidap and 1735 MΩ in 3 mM Ba^{2+}, respectively, for the cell shown in Fig. 3D).

3.4. Tenidap did not act intracellularly

Tenidap is a weak acid and has been shown to cross the membrane at physiological pH (McNiff et al., 1994). It was therefore possible that extracellularly applied tenidap might increase hKir2.3 current by acting at an intracellular site. To test for this possibility, we performed whole-cell and outside-out patch experiments in the presence of bovine serum albumin. Tenidap was reported to bind extensively to these serum proteins (Proudman and McMillan, 1991), which significantly lowers its concentration. As shown in Fig. 4A, 10 μM extracellular bovine serum albumin alone had no effect on hKir2.3 current. In the continued presence of bovine serum albumin, extracellularly applied tenidap (10 μM) was ineffective in increasing the current, consistent with the idea that the concentration of free tenidap was low in the presence of bovine serum albumin. We then investigated the sidedness of the tenidap action using outside-out patches. To this end, we included bovine serum albumin (10 μM) in the pipette solution in order to scavenge tenidap that might have permeated through the plasma membrane to the intracellular side. As shown in Fig. 4B and C, intracellular bovine serum albumin had no effect on the degree of potentiation by extracellular tenidap (30 μM), suggesting that the site of the tenidap action on hKir2.3 is not intracellular.

3.5. Potentiation of hKir2.3 by tenidap was unlikely to be secondary to pathways of arachidonic acid or protein kinase C

Tenidap has been reported to increase the concentration of intracellular Ca^{2+} by discharging intracellular Ca^{2+} stores (Fujii et al., 1995). This could lead to Ca^{2+}-dependent activation of phospholipase A_2, which triggers release of arachidonic acid. Since arachidonic acid opens hKir2.3 (Liu et al., 2001), we were interested to see if the tenidap effect on hKir2.3 might be secondary to the liberation of arachidonic acid. To this end, we compared the tenidap potentiation of $^{86}Rb^+$ efflux through hKir2.3 in the absence and presence of 4BPB (10 μM), a phospholipase A_2 inhibitor. As shown in Fig. 5A, the levels of potentiation by tenidap were not different under the two conditions, indicating that phospholipase A_2 was unlikely involved. Furthermore, arachidonic acid metabolism is also unlikely a factor since inhibitors of the cyclooxygenase [indomethacin (30 μM) and ETYA (100 μM)], lipoxygenase [NDGA (50 μM) and ETYA (100 μM)] and cytochrome P450-dependent epoxygenase [ETYA (100 μM)] pathways had no effect on the tenidap potentiation of $^{86}Rb^+$ efflux or current through hKir2.3 (Fig. 5A,B). Finally, two protein kinase C inhibitors, H7 (30 μM) and Ro 31-8220 (3 μM), also did not alter potentiation of $^{86}Rb^+$ efflux by tenidap (Fig. 5A).

4. Discussion

We have shown that the novel anti-rheumatic agent, tenidap, is a potent opener of the cloned human Kir2.3 channel. This is supported by the observation that tenidap potently (a) increased $^{86}Rb^+$ efflux and K^+ currents through hKir2.3 channels and (b) lowered the membrane potential

and input resistance in a Ba^{2+}-sensitive manner in cells heterologously expressing hKir2.3. Tenidap has little or no effect on the other cation channels tested, including Kv1.5, μ1 Na^+ channel, as well as another member of the Kir2.0 subfamily, hKir2.1, which shares ~60% amino acid identity with hKir2.3. As with arachidonic acid (Liu et al., 2001), tenidap does not appear to have an intracellular site of action.

Tenidap has been reported to increase $[Ca^{2+}]_i$ by discharging intracellular Ca^{2+} stores. This could potentially activate Ca^{2+}-dependent phospholipase A_2, which in turn liberates arachidonic acid. Indeed, low concentrations of tenidap have been shown to increase arachidonic acid release in activated macrophages (Bondeson and Sundler, 1994). Because arachidonic acid activates hKir2.3, it is tempting to ask whether hKir2.3 activation by tenidap is due to tenidap-induced arachidonic acid release or other intracellular signal transduction mechanisms. Several lines of evidence argue against these possibilities. First, high concentrations of tenidap (e.g., 30 μM as used in our experiments) that maximally activate hKir2.3 actually inhibit arachidonic acid release (Bondeson and Sundler, 1994). Second, the ability of tenidap to open hKir2.3 was not affected by 4BPB, a phospholipase A_2 inhibitor. Third, tenidap was effective in outside-out patches, which were free of intracellular Ca^{2+} (the pipette contained 5 mM EGTA), ATP and other water-soluble cytosolic factors. In addition, the tenidap action does not appear to involve the metabolic cascade of arachidonic acid (tenidap itself being a potent cyclooxygenase inhibitor) or the protein kinase C pathway. Thus, arachidonic acid or signal transduction mechanisms that depend upon such factors as Ca^{2+} do not appear to mediate the tenidap response. Although indirect effects by other intracellular second messengers are possible, they seem less likely given the fast actions of tenidap (Fig. 2B).

The tendency of tenidap to lower intracellular pH cannot explain its action on hKir2.3 since (a) there should be little change in pH_i in our whole-cell and outside-out patch experiments due to the presence of 10 mM HEPES, and (b) intracellular acidification *decreases*, rather than increases Kir2.3 current (Zhu et al., 1999).

Our results in this study identify tenidap as a potent, reversible and possibly selective opener of hKir2.3. It is conceivable that such an opener could be a valuable tool in understanding physiological and pathological processes in which Kir2.3 plays a part. In light of the critical role that Kirs play in setting the resting membrane potential and firing pattern in a variety of cell types, opening of Kir2.3 may be important for decreasing cellular excitability. This may be of particular significance in tissues of the brain and heart where Kir2.3 is abundantly expressed (Périer et al., 1994).

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