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Potentiation of the Kv1 Family K^+ Channel by Cortisone Analogues

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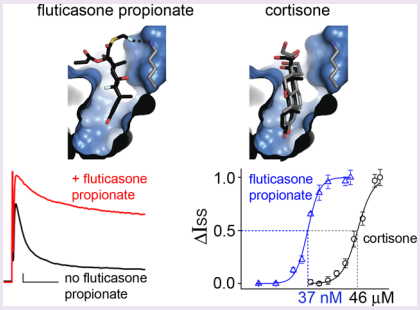
ABSTRACT: The Kv1 family voltage-dependent K^+ channels are essential for termination of action potentials in neurons and myocytes. These channels form a stable complex with their beta subunits (Kvβ), some of which inhibit channel activity. Cortisone potentiates Kv1 channel by binding to Kvβ and promoting its dissociation from the channel, but its half-maximum effective concentration is ~46 μM. To identify corticosteroids that are more efficient than cortisone, we examined 25 cortisone analogues and found that fluticasone propionate potentiates channel current with a half-maximum effective concentration (EC_{50}) of 37 ± 1.1 nM. Further studies showed that fluticasone propionate potentiates channel current by inducing dissociation of Kvβ, and docking of fluticasone propionate into the cortisone binding site reveals potential interactions that enhance the EC_{50} value. Thus, fluticasone propionate provides a starting point for rational design of more efficient small-molecule compounds that increase Kv1 activity and affect the integrity of the Kv1-Kvβ complex.



Voltage-dependent potassium channels (Kv) are tetrameric integral membrane proteins that allow K^+ to flow out of a cell upon membrane depolarization. The efflux of K^+ brings the membrane potential back to the resting value, thus reducing the cell's excitability. In humans, reduced Kv1 channel activity due to heterozygous loss-of-function mutations has been linked directly to epilepsy and atrial fibrillation,[1,2] and in theory, Kv1 channel openers could decrease hyperexcitability found in these diseases. However, currently there are no clinically useful Kv1 channel openers.

Kv1 channels assemble with a cytosolic beta subunit (Kvβ) to form a stable complex with a stoichiometry of $(Kv1)_4(Kv\beta1)_4$.[3−8] The functions of Kvβ are at least 3-fold. First, Kv1 and Kvβ associate in the endoplasmic reticulum (ER) at an early stage of channel biosynthesis,[9,10] and an intact Kv1-Kvβ complex is exported to its final destination on the plasma membrane more efficiently than Kv1 alone.[9,11,12] Second, Kvβ has a large impact on channel activities. There are three mammalian Kvβ genes, Kvβ1−3.[13] Kvβ1 and Kvβ3 have flexible N-termini that block the channel,[14] a mechanism commonly known as N-type inactivation.[15,16] Third, and perhaps most intriguingly, all three Kvβs share a highly conserved and structured core region[17,18] that acts as a functional aldo-keto reductase with NADPH as a cofactor.[19−21] Oxidation of the NADPH on the conserved core of Kvβ reduces N-type inactivation.[19,20] However, the physiological function of this redox regulated channel activity remains a mystery.

Although the Kv1-Kvβ complex is stable under physiological conditions, cortisone induces dissociation of Kvβ from the channel by binding to Kvβ at a site close to the Kv1-Kvβ interface.[22] When cortisone was applied to the Kv1-Kvβ1 complex, a significant increase of current was observed due to loss of N-type inactivation, and the effect had a half-maximum effective concentration (EC_{50}) of 46 ± 1.1 μM.[22] In addition to being a Kv1 channel opener, cortisone could be a chemical knockout reagent to facilitate investigation of Kvβ functions in physiology. Eliminating Kvβ after the Kv1-Kvβ complex is already delivered to its intended position has an advantage over genetic knockout of Kvβ genes, because the chemical approach will likely preserve the location and surface expression level of the channel. As the first step to improving the affinity and efficacy of cortisone, we examined how cortisone analogues affect channel current.

Modulation of Kv1 Current by Cortisone Analogues. A total of 25 commercially available cortisone analogues were examined, and the analogues were perfused to inside-out patches expressing Kv1.1 and Kvβ1. A single concentration of 500 μM was used for the majority of the analogues, except for cortisone 21-acetate and fluticasone propionate, which were 100 and 20 μM in the initial test due to low solubility. Change in channel current after perfusion of a compound was quantified and plotted in Figure 1. Typical currents before and after perfusion are shown for the vehicle control (Figure 1a), cortisone (Figure 1b), cortisone 21-acetate (Figure 1c), and fluticasone propionate (Figure 1d). Only two compounds, cortisone 21-acetate and fluticasone propionate, potentiated channel current significantly (Figure 1e and f).

To further characterize the effect of the two compounds, channel current was recorded at various concentrations of either cortisone 21-acetate or fluticasone propionate, and

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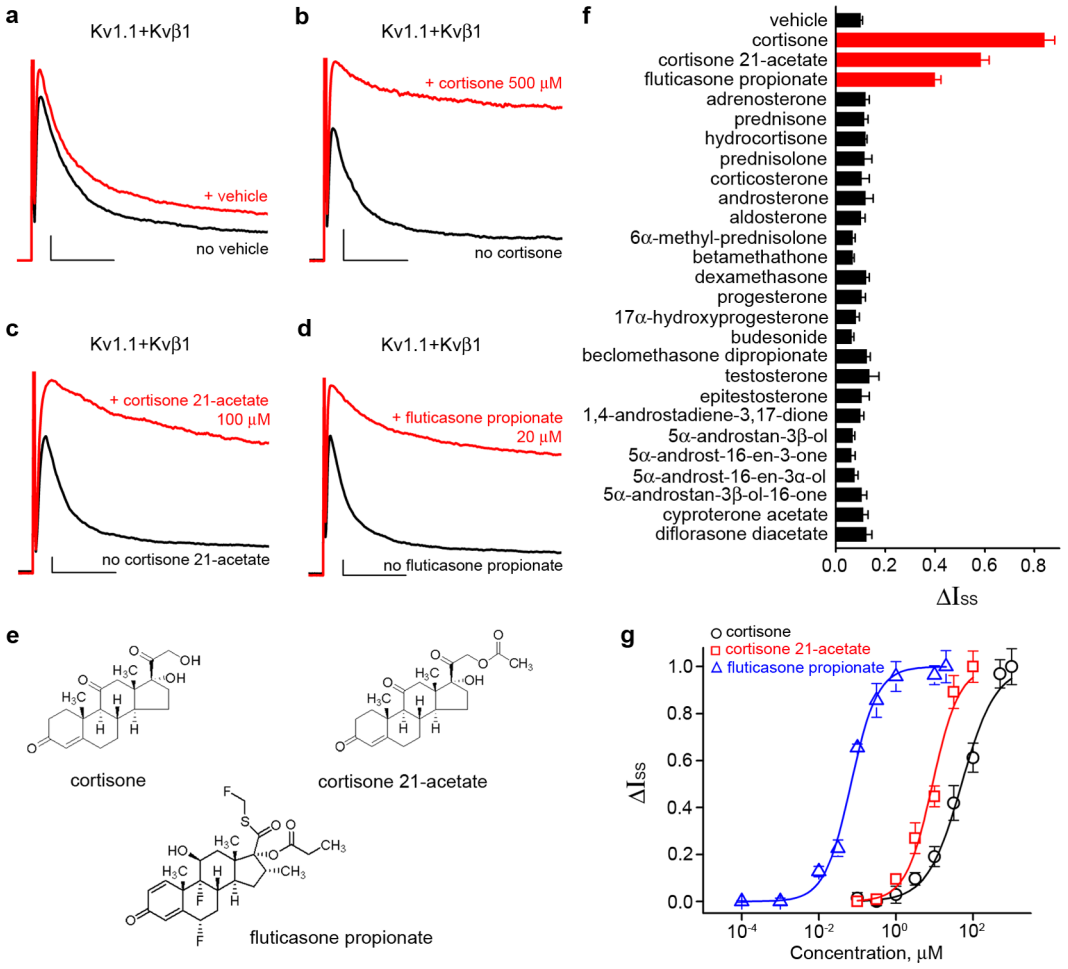

Figure 1. Potentiation of Kv1 current by cortisone analogues. (a–d) Current traces of Kv1.1 co-expressed with Kvβ1 before (black) and after (red) perfusion of the vehicle (1% DMSO) (a), 500 μM cortisone (b), 100 μM cortisone 21-acetate (c), or 20 μM fluticasone propionate (d). Scale bars represent 300 pA and 10 ms. (e) Chemical structures of cortisone, cortisone 21-acetate, and fluticasone propionate. (f) ΔI_{ss} after perfusion of the vehicle (1% DMSO), cortisone and 25 cortisone analogues. (g) Normalized ΔI_{ss} induced by different concentrations of cortisone (○), cortisone 21-acetate (□), and fluticasone propionate (△) in Kv1.1 co-expressed with Kvβ1. The solid curves are the dose–response function fit to the data points. Error bars are SEM from 3 to 13 patches.

dose–response profiles were constructed (Figure 1g), which led to an estimated EC_{50} of 9.0 ± 1.1 μM and 37 ± 1.1 nM for cortisone 21-acetate and fluticasone propionate, respectively. Although the EC_{50} value for cortisone 21-acetate is slightly lower than that of cortisone (46 μM), that of fluticasone propionate is substantially lower.

Mechanism of Kv1 Current Potentiation. To examine if potentiation of current in the presence of cortisone 21-acetate or fluticasone propionate is mediated by the conserved core of Kvβ1, a mutant Kv1.1, Kv1.1-inact was tested. The Kv1.1-inact construct contains the inactivation gate of Kvβ1 spliced onto the N-terminus of Kv1.1, and therefore lacks the core domain while still generating inactivating currents similar to these produced by co-expression Kv1.1 and Kvβ1[19,22] (Figure 2a). When perfused to the inside-out patches expressing Kv1.1-inact, neither cortisone 21-acetate nor fluticasone propionate increased current significantly more than the vehicle control (Figure 2a and d), indicating that both compounds potentiate channel current through the conserved core of Kvβ1.

Although cortisone 21-acetate has a pregnene ring identical to that of cortisone, fluticasone propionate does not, and this raises the question of whether the two compounds bind to Kvβ1 similarly to cortisone. A previous study showed that Kvβ provides a deep binding pocket to accommodate the pregnene ring of cortisone, and a mutation in the binding pocket, V245R, eliminates cortisone binding.[22] We therefore examined how the compounds affect the V245R mutant protein. Both compounds induced a small change in channel current, which was not significantly different from the vehicle controls (Figure 2b and d). These results confirmed that the two compounds likely interact with Kvβ1 at the same site where cortisone binds.

To find out if cortisone 21-acetate or fluticasone propionate potentiate channel current by promoting dissociation of Kvβ1, we tested the two compounds on the Kvβ1-Kv1.1 chimera, in which Kvβ1 was spliced to the N-terminus of Kv1.1 and thus cannot dissociate from the channel. The chimera was not potentiated by either of the compounds, indicating that both compounds modify channel current by inducing dissociation of Kvβ1 (Figure 2c and d).

To further demonstrate that cortisone 21-acetate or fluticasone propionate binds to Kvβ1 at the same site as cortisone, we used a scintillation proximity assay (SPA) to measure the binding of radio-labeled cortisone to Kvβ. By competing with the labeled cortisone with various concentrations of unlabeled cortisone, an equilibrium inhibition constant (K_i) was obtained at 30 ± 10 μM (Figure 2e). The

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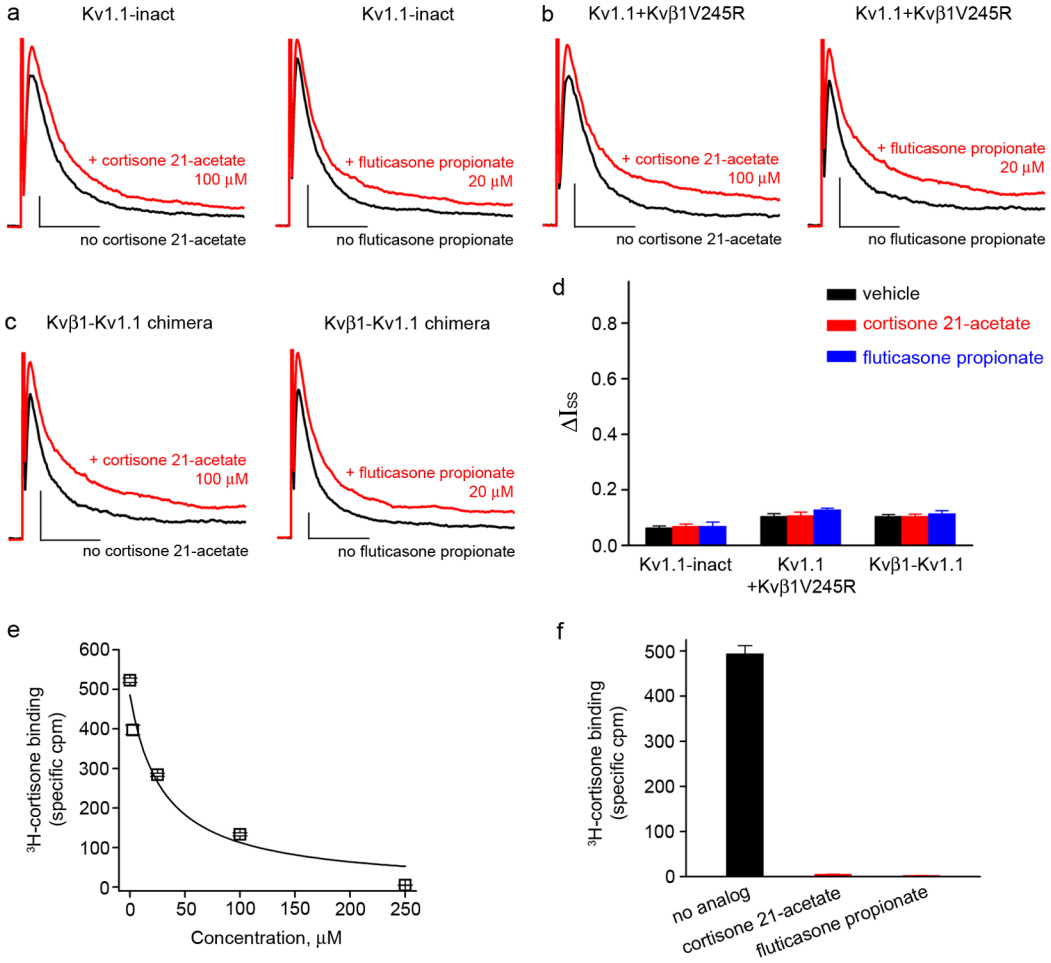

Figure 2. Mechanism of channel potentiation by cortisone 21-acetate and fluticasone propionate. (a–c) Current traces of Kv1.1-inact (a), Kv1.1 co-expressed with Kvβ1 V245R (b), and Kvβ1-Kv1.1 chimera (c) before (black) and after (red) perfusion of 100 μM cortisone 21-acetate (left panel) or 20 μM fluticasone propionate (right panel). Scale bars represent 300 pA and 10 ms. (d) ΔI_{ss} after perfusion of the vehicle (1% DMSO), cortisone 21-acetate, or fluticasone propionate. Error bars are SEM from 3 to 5 patches. (e) SPA-based ^{3}H-cortisone equilibrium binding with increasing concentrations of cortisone. The solid curve corresponds to data fit with a single-site binding isotherm. Error bars are SEM from 3 measurements. (f) SPA-based ^{3}H-cortisone equilibrium binding without or with cortisone analogues. Error bars are SEM from 3 measurements.

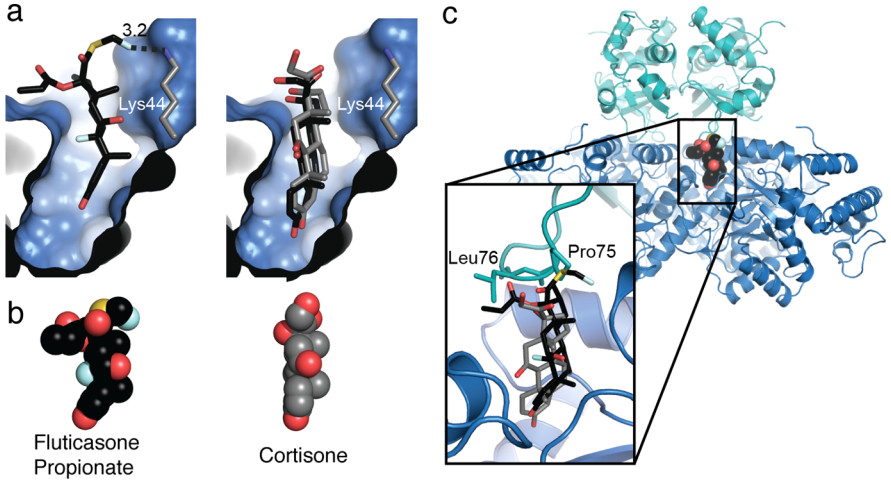

Figure 3. Comparison of cortisone and fluticasone propionate binding to Kvβ. (a) Surface representation of the Kvβ binding site shown with either the docking model of fluticasone propionate (left) or cortisone (right) from the crystal structure (gray), overlaid with a docking model produced by the same protocol used for the fluticasone propionate model (black). (b) Fluticasone propionate (left) and cortisone (right) shown as spheres. (c) The location of the binding site on the Kvβ tetramer (blue) shown in complex with the channel T1 domain (teal, PDB 1EXB). The inset shows expected clashes between cortisone (gray) or fluticasone propionate (black) and residues 75–76 on the T1 domain.

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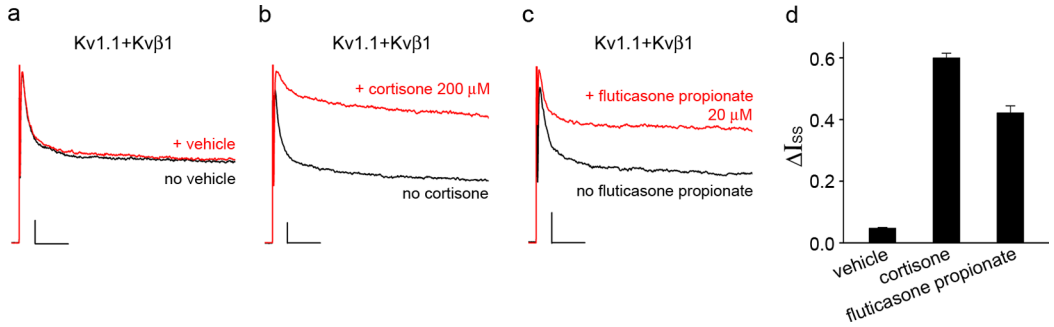

Figure 4. Cortisone and fluticasone propionate potentiate Kv1 current in HEK cells. Current traces of Kv1.1 co-expressed with Kvβ1 from whole-cell patch clamped HEK cells before (black) and after (red) perfusion of the vehicle (1% DMSO) (a), 200 μM cortisone (b), or 20 μM fluticasone propionate (c). Scale bars represent 300 pA and 30 ms. (d) ΔI_{ss} after perfusion of the vehicle (1% DMSO), cortisone, or fluticasone propionate. Error bars are SEM from 3 to 5 patches.

value is comparable to the EC_{50} values obtained from measuring dissociation of Kvβ from the channel.[22] Unlabeled cortisone 21-acetate (250 μM) and fluticasone propionate (250 μM) reduced cortisone binding by 95 ± 0.2% and 98 ± 0.1%, respectively (Figure 2f). These data provide another line of evidence that the two identified cortisone analogues bind to the same binding pocket as cortisone.

Interactions of Fluticasone Propionate with Kvβ. To determine how fluticasone propionate interacts with Kvβ to achieve an EC_{50} almost 1000 fold smaller than that of cortisone, we attempted to obtain a structure of fluticasone propionate in complex with Kvβ, but our repeated efforts were unsuccessful. We therefore used the program AutoDock Vina[23] to model fluticasone propionate into the known binding site for cortisone. The lowest energy conformation is shown in Figure 3a. As a control, an identical docking protocol was performed with cortisone, which predicted a conformation for the ligand with a 0.9 Å root-mean-square deviation from the crystallographically determined position (Figure 3a, right). While the fluticasone propionate model suggests some possible electrostatic interactions with the protein not present with cortisone, including a hydrogen bond between a fluorine atom and residue Lys44 on the protein, a more significant contributor to the lower EC_{50} of fluticasone propionate may be the improved shape complementarity between the ligand and binding site. The fluorine atoms on fluticasone propionate appear to increase the area of the interacting surface between the ligand and the binding pocket (Figure 3a). Also, the additional double bond between carbons 1 and 2 introduces a kink to the steroid rings that better matches the sock-like shape of the cavity (Figure 3b).

As shown in Figure 3c, cortisone's position in the binding pocket on Kvβ results in steric clashes between the D ring and the hydroxyl group on the 17th position and residues 75 and 76 on the T1 domain of the channel, leading to dissociation of Kvβ. Fluticasone propionate has two large substitutions at the 17th position, resulting in an increased volume protruding into the Kvβ/T1 interface. The more severe steric clash generated by the presence of fluticasone propionate may contribute to its lower apparent EC_{50}.

Cortisone and Fluticasone Propionate Potentiate Kv1 Current in HEK Cells. Since all electrophysiological studies have been performed on inside-out patches with cortisone and fluticasone propionate being perfused directly to the intracellular side of the membrane, as a first step to demonstrate the utility of these compounds, we examined them on HEK293 cells stably transfected with Kv1.1 and Kvβ1. The whole-cell patch clamp configuration was employed, and the compounds were applied to the extracellular side of a cell. Cortisone and fluticasone propionate both potentiated channel current significantly (Figure 4a–d), indicating that both could be effective on primary cells or in an organism.

Discussion. In summary, we have found that fluticasone propionate potentiates Kv1 current by promoting dissociation of Kvβ1. Compounds that target Kvβ have specificity to Kv1 channels because of exclusive assembly between Kv1 and Kvβ.[3,8,24] The EC_{50} of the fluticasone propionate effect is significantly smaller than that of cortisone, and the improvement can be attributed, at least in part, to favorable interactions between fluorine on the ligand and a positively charged residue on the protein and to better shape complementarity between the ligand and the binding pocket. It is intriguing that although fluticasone propionate has a much lower EC_{50} value, its maximum effect on ΔI_{ss} is smaller than that of cortisone (Figure 1d,f). Nevertheless, all three corticosteroids are highly effective in potentiating channel current, and fluticasone propionate represents an improved starting point over cortisone for further structure–activity studies to identify compounds that have better efficiency to potentiate Kv1 channel current.

Fluticasone propionate has long been used as an inhaled drug to treat asthma, and because it is an analogue of corticosteroid, its mechanism of action is thought to be related to the regulation of the immune response. However, the current study suggests that fluticasone propionate may play an additional role: Kv1 and Kvβ1 are both highly expressed on airway smooth muscle cells,[25] and the inhaled fluticasone propionate could potentiate these channels in the smooth muscle, which would lead to relaxation of the smooth muscle cells and relief of airway obstruction.

METHODS

Molecular Biology and Electrophysiological Recordings. For oocyte expression, the full length cDNA of rat Kv1.1 (accession number NM_173095) or that of rat Kvβ1 (accession number NM_017303) was cloned into a modified pBluescript vector (a gift from Dr. Mark Sonders, Columbia University) between the *Kpn*I and *Eco*RI sites for *in vitro* transcription. The Kv1.1-inact or the Kvβ1-Kv1.1 connected chimera was generated by splicing the DNA sequence encoding residues 1 to 70 of Kvβ1 or the full length of Kvβ1 onto that of Kv1.1 (encoding residues 2 to 495-stop) by the overlapping PCR method, respectively. The PCR products were then ligated into the same pBluescript vector. Point mutations were made using the QuikChange kit (Agilent Technologies). mRNA was

prepared by *in vitro* T7 polymerase transcription after DNA was linearized with NotI. mRNAs were purified using the Trizol reagent (Invitrogen Inc.) and injected into Xenopus oocytes for channel expression. For co-expression, mRNAs of Kv1.1 and Kvβ1 were mixed and injected together into oocytes. The inside-out patch clamp recordings followed the same procedures detailed in refs 19 and 20.

To prepare HEK293 cells stably transfected with Kv1.1 and Kvβ1, the cDNA of Kv1.1 was cloned into a pcDNA5/FRT/TO vector and that of Kvβ1 was cloned into a pIRESpuro vector (both from Invitrogen). The Flp-in HEK293 cell line was used for transfections and the stably transfected cell lines were maintained under three antibiotics, blasticidin (final concentration of 15 μg/mL), puromycin (final concentration of 2 μg/mL), and hygromycin B (final concentration of 100 μg/mL). Tetracycline at the final concentration of 2 μg/mL was added 18−24 h before patch clamp experiments to induce channel expression. The glass patch pipette solution contained (in mM): K-aspartate 110, $MgCl_2$ 1, $CaCl_2$ 1, EGTA 11, HEPES 10, and ATP-K_2 5 at pH 7.3. The external solution contained (in mM): NaCl 132, KCl 4.8, $MgCl_2$ 1.2, $CaCl_2$ 2, HEPES 10 at pH 7.4, and glucose 5. K^+ currents were elicited by holding the patch at −80 mV and stepping to +60 mV for 400 ms.

Cortisone analogues were first dissolved in dimethylsulfoxide (DMSO) and then diluted into the internal buffer to the desired concentrations. The final concentration of DMSO was kept at 1% (v/v) for all analogues. Kvβ1 has a cysteine residue at position 7 that can be oxidized on inside-out patches to affect channel inactivation.[26] To eliminate concerns that the effect could be due to cysteine oxidation, we mutated the seventh position cysteine to an alanine, for Kvβ1 and Kvβ1-Kv1.1. ΔI_{ss}, the increase of steady-state current as a fraction of the initial inactivating current, was calculated by the following equation:

$$\Delta I_{ss} = \frac{I_{ss,A} - I_{ss,B}}{I_{peak,B} - I_{ss,B}}$$

where $I_{ss,A}$ and $I_{ss,B}$ are the steady-state currents, measured at 200 ms after depolarization, after and before compounds perfusion, respectively. $I_{peak,B}$ is the peak current amplitude before compounds perfusion. All chemicals were purchased from Sigma.

Scintillation Proximity Assay. The core domain of Kvβ2 was expressed and purified as described in ref 20. Kvβ2 was used instead of Kvβ1 because it is more stable biochemically, and the amino acid sequences of the proteins are more than 80% identical. It has been demonstrated previously that cortisone can bind to both Kvβ cores, and the binding promotes dissociation.[22] The Kvβ2 core used in the binding study carries a mutation, W272A, which eliminates a secondary binding site that is not involved in dissociation of Kvβ.[22]

Binding of 80 nM [1,2,6,7-^{3}H]cortisone (90 Ci/mmol; American Radiolabeled Chemicals, Inc.) to 1 μg of purified protein was measured in 100 μL of 50 mM Tris/Mes, pH 8.0, 300 mM KCl, 10% [w/v] glycerol, 100 μM tris(2-carboxyethyl)phosphine (TCEP) containing 2.5 mg Cu^{2+}-coated YSi-SPA beads/mL (Perkin-Elmer) for 4 h at 4 °C. Reactions were performed in 96-well white wall clear-bottom plates and assayed in a Wallac photomultiplier tube MicroBeta counter. Non-proximity background signals were determined for all conditions tested in the presence of 400 mM imidazole and used to determine the specific counts per minute (cpm). Appropriate concentrations of the indicated compounds were added to the reactions as indicated. All conditions were tested in triplicate.

Docking. The model of fluticasone propionate was built using the PRODRG server.[27] Coordinates from a structure of the unliganded Kvβ1/T1 complex, PDB entry 1EXB,[3] were used to build the Kvβ tetramer. Hydrogens and Gasteiger charges were added to ligand and receptor using AutoDockTools,[28] and the docking was performed using AutoDock Vina.[23] Figures of the protein and ligand structures were prepared in PyMol (Schrödinger, San Diego, CA).

Reagents. Twenty-five cortisone analogues were purchased from Sigma, including cortisone 21-acetate (C3130), fluticasone propionate (F9428), adrenosterone (284998), prednisone (286990), hydrocortisone (H4001), prednisolone (P6004), corticosterone (C2505), androsterone (219010), aldosterone (A9477), 6α-methyl-prednisolone (M0639), betamethasone (B7005), dexamethasone (861871), progesterone (P8783), 17α-hydroxyprogesterone (H5752), budisonide (B7777), beclomethasone dipropionate (B3022), testosterone (T1500), epitestosterone (E5878), 1,4-androstadiene-3,17-dione (A7505), 5α-androstan-3β-ol (A2480), 5α-androst-16-en-3-one- (A8008), 5α-androstan-3β-ol-16-one (A7386), 5α-androst-16-en-3α-ol (A7883), cyproterone acetate (C3412), and diflorasone diacetate (D8286).

Data Statistics. The Origin 7.5 software package was used for statistical analysis of the data. The results are expressed as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was used to assess changes of a mean value. A value of $p < 0.05$ was considered significant.

■ ASSOCIATED CONTENT

Accession Codes

Coordinates and structure factors of Kvβ2-cortisone[22] (3EAU) and T1-Kvβ2 complex[3] (1EXB) were downloaded from PDB. GenBank identifiers: rat Kv1.1, NM_173095; rat Kvβ1, NM_017303.

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Notes

The authors declare no competing financial interest.

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