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β-Adrenergic receptors couple to CFTR chloride channels of intercalated mitochondria-rich cells in the heterocellular toad skin epithelium

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Abstract

In the heterocellular toad skin epithelium the β-adrenergic receptor agonist isoproterenol activates cyclic AMP-dependent Cl^- channels that are not located in the principal cells. With four experimental approaches, in the present study, we tested the hypothesis that the signalling pathway targets cystic fibrosis transmembrane conductance regulator (CFTR)–chloride channels of mitochondria-rich cells. (i) Serosal application of isoproterenol ($\log_{10}EC_{50} = -7.1 \pm 0.2$; Hill coefficient = 1.1 ± 0.2), as well as noradrenaline, activated an anion pathway with an apical selectivity sequence, $G_{Cl} > G_{Br} \geq G_{NO_3} > G_I$, comparable to the published selectivity sequence of cloned human CFTR expressed in *Xenopus* oocytes. (ii) Known modulators of human CFTR, glibenclamide (200 μmol/l) and genistein (50 μmol/l), depressed and activated, respectively, the receptor-stimulated G_{Cl}. Genistein did not modify the anion selectivity. (iii) Transcellular voltage clamp studies of single isolated mitochondria-rich cells revealed functional β-adrenergic receptors on the basolateral membrane. With ~ 60,000 mitochondria-rich cells per cm^2, the saturating activation of 11.9 ± 1.6 nS/cell accounted for the measured isoproterenol-activated transepithelial conductance of 600–900 μS/cm^2. In forskolin-stimulated cells, glibenclamide (200 μmol/l) reversibly inhibited the transcellular conductance by 9.6 ± 1.6 nS/cell. (iv) With primers constructed from cloned *Xenopus* CFTR and PCR amplification of reverse-transcribed mRNA from toad skin, full-length *Bufo* CFTR cDNA was generated. The derived protein of 1466 residues shows 86% homology with *x*CFTR and 89% homology with *h*CFTR. All major functional sequences, that is, the R- and the NBF1- and NBF2-domains are well-conserved as are the predicted transmembrane segments proposed to form the pore of the channel protein. These new results taken together with our previously identified small-conductance CFTR-like Cl^- channel in the apical membrane of the mitochondria-rich cells lead to the conclusion that the toad's CFTR gene codes for a functional Cl^- channel in the apical plasma membrane of this minority cell type.

Keywords: Single-cell voltage clamp; CFTR Cl^- channel; Genistein; Glibenclamide; Cyclic AMP; Forskolin

1. Introduction

The function and regulation of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel [1] have been studied extensively in mammalian epithelia [2]. The protein is expressed in the apical membrane, and following cyclic AMP-dependent phosphorylation of the R-domain cytosolic ATP activates channel activity [3–5]. Phosphorylation by the protein kinase A (PKA) catalytic subunit was indicated in studies of native cells [6] and cultures expressing native [7,8] or cloned [9] human CFTR. In vivo, the formation of the second messenger cyclic AMP is controlled by, e.g., stimulation of β-adrenergic receptors on the basolateral membranes of the epithelium. This scheme seems to be applicable to several mammalian epithelia like ducts of sweat glands [8,10], upper airways [11], intestine [12], ducts of the exocrine pancreas [13], and male reproductive tracts [14]. Likewise, salt secretion by epithelia of lower vertebrates such as shark rectal gland [15,16], gills of marine teleosts [17,18] and frog subepidermal gland [19–21] depends on cyclic AMP-regulated CFTR. The absorbing chloride cells and small intestine of the freshwater-adapted euryhaline teleost, *Fundulus heteroclitus*, also expresses CFTR. However, in contrast to the seawater-adapted *Fundulus*, the channel protein in the freshwater-adapted fish is localized to the basolateral membrane [18]. The purpose of the present study is to investigate a previously identified β-adrenergic receptor-regulated chloride conductance of the skin of anuran Amphibia, which was hypothesized to be located in mitochondria-rich cells of

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the NaCl- and fluid-absorbing epidermis [22]. This was done by selectivity and pharmacological analysis of the activated channel of the intact epithelium, by transcellular voltage clamp of single isolated mitochondria-rich cells, and by molecular biological characterization of the putative channel protein. Some of the results have been referred to in previous review articles [23,24].

2. Materials and methods

2.1. Animals and preparations

Toads (*Bufo bufo*) were kept in an indoor (winter) or outdoor terrarium with free access to a pool of tap water and fed with mealworms ad lib. For increasing the yield of mitochondria-rich cells in single-cell studies, toads were kept for 1–2 weeks in running distilled water (20 °C). The animals were killed by double pithing and the skin removed by dissection. The epithelium was isolated by exposure of the serosal side of the skin for 2 h at room temperature by a Ringer's solution containing collagenase at a concentration of 2 mg/ml (Type 2, Worthington Chemical, NJ). Single mitochondria-rich cells were obtained by trypsin treatment of the isolated epithelium by a method modified from Refs. [25,26]. For 1 h, the isolated epithelium was kept in collagenase Ringer (2 mg/ml, C-9891, EC 3.4.24.3, Sigma, St. Louis, MO, USA) and after washing in Ca^{2+}-free Ringer, it was bathed in Ca^{2+}-free trypsin Ringer (0.1 mg/ml, T-4665, EC 3.4.21.4, Sigma) and centrifuged for 5 min at 800–1200 rpm. The remaining epithelium was transferred to a fresh trypsin solution, while the isolated cells were washed and stored in NaCl Ringer's solution until use. Rounds of 5 min trypsin treatment of the epithelium, centrifugation, and wash were repeated until the major fraction of the epithelial cells was isolated. In order to protect the β-adrenergic receptors from exogenous enzymes, in all steps of the isolation procedure, the solutions contained 1 μmol/l of the receptor antagonist propranolol.

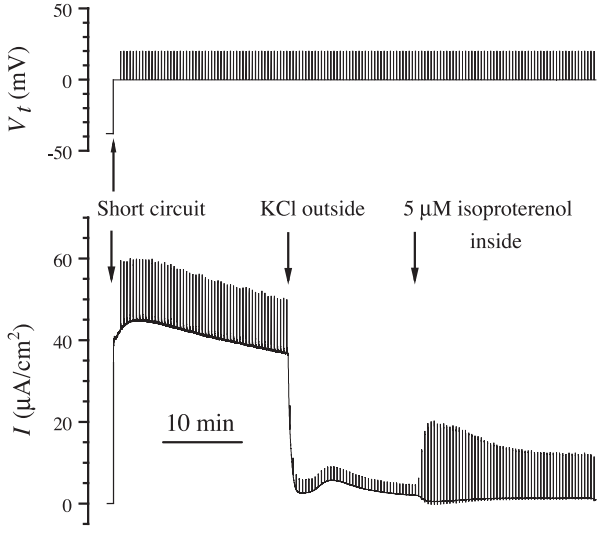


Fig. 1. The protocol for studying the receptor-activated chloride conductance of collagenase-isolated toad skin epithelium. When mounted with NaCl–Ringer's solution on both sides, the preparation exhibited a spontaneous transepithelial potential difference, $V_t = -38$ mV. Following short-circuiting (arrows), for monitoring the conductance at intervals of 20 s, V_t was clamped to +20 mV for 2 s. At the arrow indicated 'KCl' the outside of the epithelium was superfused with a solution in which all of the sodium ions were replaced by potassium ions, which reduced the short-circuit current as well as the conductance. Adding isoproterenol to the serosal solution stimulated the transepithelial conductance leaving I_{sc} near zero.

Table 1
Transepithelial bioelectric parameters of toad skin collagenase-isolated epithelium exposed to NaCl Ringer's solution on both sides

	V_t (mV)	R_t (KΩ·cm^2)	I_{sc} (μA/cm^2)	N
Mean ± S.E.	−21.0 ± 1.5	1.191 ± 0.103	22.0 ± 1.9	49

V_t and R_t are the steady state 'open-circuit' transepithelial electrical potential difference (inside solution grounded) and resistance, respectively. I_{sc} is the steady state short-circuit current ($V_t = 0$ mV). N = number of preparations.

2.2. Experimental setups

For studies of mitochondria-rich cells in situ, the intact epithelium was mounted in a Perspex chamber that could be perfused continuously on the outside with well-aerated Ringer's solutions of different compositions as indicated in the text. Isolated cells were transferred to a Perspex chamber with a cover-glass bottom and volume of 300 μl. By gravity force, the chamber was perfused at a rate of ~ 85 μl/s with a well-aerated solution of desired composition. With the chamber mounted on the table of a Nikon TMS inverted microscope (DFA A/S, Copenhagen, Denmark) the cells were viewed at ×40 magnification. By suction, the neck of a mitochondria-rich cell was positioned in the tip of a patch clamp pipette (Vitrex, Modulohm A/S Herlev, Denmark) fabricated on a two-step vertical puller (Hans Ochotzki, Homburg, Germany) and polished (MF-90 Narishige, Tokyo, Japan) for obtaining a resistance of 1.2–1.6 MΩ (measured with NaCl Ringer's in bath and pipette).

2.3. Electrophysiological measurements

The transepithelial potential difference, V_t, was measured via calomel electrodes matched to within 1 mV (K401, Radiometer, Copenhagen). Current was passed across the preparation via Ag/AgCl half-cells placed in the chambers. In

Table 2
Transepithelial electrical conductance of the isolated epithelium exposed to NaCl–Ringer's solution on the inside and a Na^+-free Ringer's solution on the outside

	Control	Isoproterenol stimulated		
	G_t (μS/cm^2)	G_t (μS/cm^2)	I_{sc} (μA/cm^2)	N
Mean ± S.E.	292 ± 51	1098 ± 63	0.3 ± 0.3	25

Table 3
Conductance with gluconate outside (G_{leak}), receptor-activated chloride conductance (G_{Cl}), and relative selectivity of the receptor-activated conductance

		G_{leak} (μS/cm^2)	G_{Cl} (μS/cm^2)	G_{Br}/G_{Cl}	G_{NO_3}/G_{Cl}	G_I/G_{Cl}
1-iso	Mean ± S.E.	166 ± 22	598 ± 97	0.67 ± 0.04	n.d.	0.18 ± 0.03
	N	6	6	6	–	6
2-iso	Mean ± S.E.	190 ± 18	898 ± 125	0.73 ± 0.01	0.64 ± 0.04	0.20 ± 0.01
	N	9	9	9	9	9
3-iso	Mean ± S.E.	274 ± 46	870 ± 301	0.67 ± 0.05	0.62 ± 0.14	0.27 ± 0.02
	N	5	5	5	5	5
noradr	Mean ± S.E.	161 ± 20	853 ± 138	0.64 ± 0.04	0.43 ± 0.09	0.17 ± 0.04
	N	6	6	6	6	6

The numbers given in columns 4–7 are corrected for the estimated leak conductance according to Eq. (2) in the text.
Three series of experiments were conducted with 5 μM isoproterenol in the serosal bath (1-iso, 2-iso, and 3-iso, respectively). In the 1-iso group, 100 μmol/l furosemide was contained in the serosal solution. In this one shift to nitrate solution was not included in the protocol (n.d. = not determined).
In the fourth series of experiments, 10 μM noradrenalin was added to the serosal bath (noradr).
In the 3-iso group, the conductance was measured by a transepithelial voltage pulse with amplitude, $\Delta V_t = -20$ mV. In the other three groups, the conductance was determined by $\Delta V_t = +20$ mV as illustrated in Fig. 1.

experiments in which the anion composition of the outside solution was changed, a platinum wire replaced the Ag/AgCl current electrode of the outside chamber. Voltage clamping at $V_t = 0$ mV was performed by a VCC600 amplifier (Physiological Instruments Inc., San Diego). The transepithelial conductance was measured at intervals of 20 s by recording the current response to a voltage pulse of 20 mV of amplitude and 2-s duration. The amplifier was interfaced to a PowerLab/8SP A/D converter and the digitized signals processed with the Chart software (ADInstruments, Castle Hill, NSW, Australia). Single-cell voltage clamp was performed with the Axopatch-200B amplifier interfaced to a Digidata 1322A using pClamp9 for recording and analysis (Axon Instruments, Foster City, CA). Currents were low-pass filtered (20 Hz corner frequency, Frequency Device, MA, USA) and sampled at a rate of 50 Hz.

2.4. Cloning of toad CFTR

By sequence comparison of CFTR from different species, three primers were generated using *x*CFTR cDNA [27] as template. The primers (MWG-Biotech) were designed to contain restriction endonuclease sites at their 5′-ends, enabling us to clone the fragments into a vector for sequencing. Total RNA was isolated from belly skin of a toad using a guanidine thiocyanate-phenol extraction kit from Advanced Biotechnologies, Ltd. OneStep RT-PCR (Qiagen) was performed, the PCR products displayed on a 1% agarose gel and bands were cut out and eluted. The amplified products was then digested with restriction endonucleases and cloned into the pEGFP-C2 vector (Clontech), mini-prepped (Qiagen), and sequenced. Alignment of *bb*CFTR with other CFTR's was performed using Vector NTI (Informax Inc.).

2.5. Composition of solutions and chemicals

The conventional amphibian Ringer's solution had the following composition (mM): 118.3 Na^+, 1.9 K^+, 1.0 Ca^{2+}, 0.5 mM Mg^{2+}, 115.8 Cl^-, 5 acetate, 2.4 HCO_3^-, pH = 8.1 when gassed with atmospheric air. This solution was used on the serosal side in whole-tissue studies and as bath and pipette solution in studies of single cells. For eliminating currents through apical Na^+ channels, Na^+ in the outside bathing solution was replaced by K^+ in studies of intact epithelia, and 100 μmol/l amiloride was added to the pipette solution in single cell studies. In the anion selectivity study, chloride from the above solution was replaced mole for mole by bromide, nitrate, iodide and gluconate, respectively. Amiloride, dibutyryl cyclic AMP, dimethylsulfoxide (DMSO), furosemide, glibenclamide (Glybenclamide), genistein, isoproterenol, noradrenalin (± Arterenol), and DL-propranolol were from Sigma-Aldrich. Isoproterenol solu-

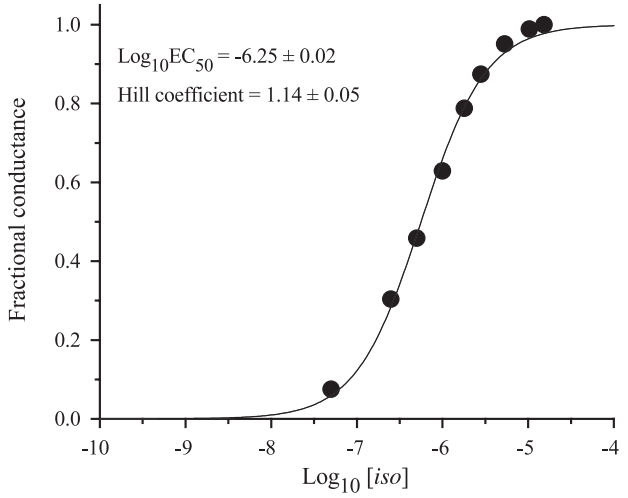


Fig. 2. Example of dose–response relationship of the isolated epithelium. The conductance of the preparation is expressed relative to the saturating conductance obtained with 10 μmol/l and with the baseline conductance observed prior to addition of isoproterenol subtracted. The theoretical curve is the best fit of Eq. (1) of the text to the experimental values with the errors indicated of the estimates of the two free variables. The fit was generated by the Simplex routine of Origin© ver. 7. Seven preparations were investigated with this protocol with the mean ± S.E. indicated in the text.

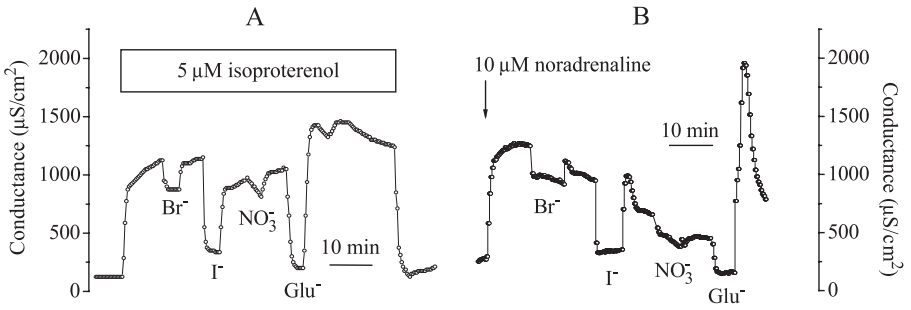


Fig. 3. The anion selectivity sequence of the receptor-activated chloride conductance. (A) With Cl^- in the external perfusate stimulation by 5 μmol/l isoproterenol in the serosal bath increased the conductance from 180 to 1125 μS/cm^2. The activated conductance depended on the anion of the external perfusate as indicated. The conductance with NO_3^- on the outside was taken as the value recorded prior to return to Cl^-. With gluconate, return to Cl^- resulted in a transient conductance stimulation (see also B). Wash with agonist-free solution on the serosal side returned the conductance to its initial low value of about 200 μS/cm^2. (B) A similar protocol was applied for the study of the transepithelial conductance with noradrenalin in the serosal bath.

tions were made immediately before use, and glibenclamide and furosemide solutions were made from stock solutions of 100 and 10 mmol/l DMSO, respectively.

3. Results

3.1. Conductance activation of the intact epithelium by receptor stimulation

Routinely, the transport activity of the isolated epithelium was analysed by monitoring the transepithelial potential difference (V_t) and active sodium current with NaCl–Ringer's solution on both sides. Fig. 1 shows the standard protocol applied to a preparation with a steady state $V_t = -38$ mV and an active Na^+-current, $I_{sc} \approx 40$ μA/cm^2. Mean values for 49 isolated epithelia are given in Table 1. For eliminating the active sodium current, about 1 h after mounting of the preparation in the perfusion chamber, the external side was superfused with a KCl–Ringer's solution, and at steady state, 5 μM isoproterenol was added to the serosal bath. Following receptor occupation, the transepithelial conductance increased and attained a quasi-stationary value in the course of 15–25 min. Application of the agonist resulted in a significant conductance increase while the short-circuit current was maintained at a value not significantly different from zero (Fig. 1, Table 2). This provides the evidence that the conductance increase is not caused by stimulation of functional subepidermal glands, which would generate a significant inward short-circuit current carried by an active efflux of Cl^- [28,29]. A similar conclusion was reached in a study with the membrane-permeable dibutyryl cyclic AMP analogue, which stimulated the conductance of amiloride-treated preparations while the short-circuit current remained zero ($I_{sc} = 0.1 \pm 0.3$ μA/cm^2 with 500 μM db-cyclic AMP [22]). It is also in agreement with a light microscopic examination of the collagenase-isolated epithelium, showing that the isolation protocol with collagenase removes the subepidermal glands leaving their ducts closed

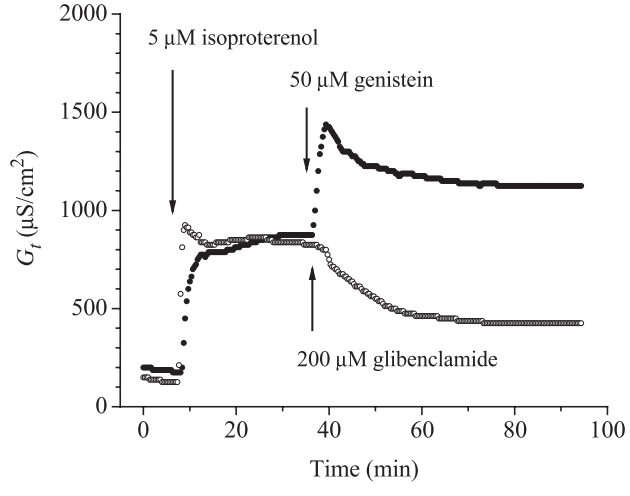


Fig. 4. Response of the β-adrenergic receptor-activated Cl^- conductance to modulators of the activity of CFTR Cl^- channels. Genistein (50 μmol/l) added to the external perfusion solution resulted in fast stimulation of the conductance. Glibenclamide added to the serosal bath at concentration of μmol/l slowly decreased the receptor-activated Cl^- conductance.

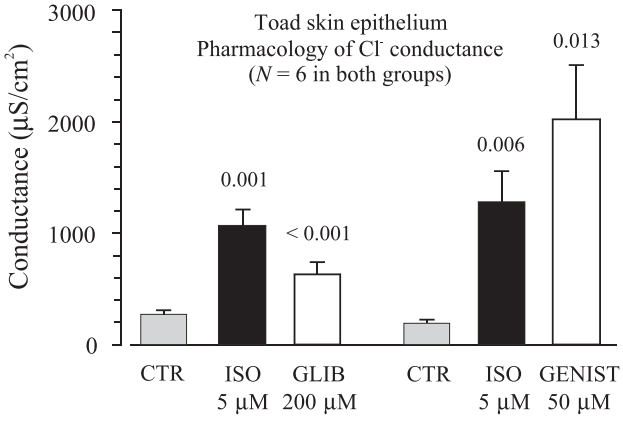


Fig. 5. Summary of the pharmacology of the β-adrenergic receptor-activated Cl^- conductance. Mean ± S.E. are indicated with levels of significance given for stimulation by isoproterenol, and subsequent modulation of the conductance by glibenclamide or genistein, respectively.

Table 4
Anion selectivity sequence of the receptor-activated conductance following stimulation by 50 μmol/l genistein added to the outer solution

	Protocol (5 μmol/l isoproterenol + 50 μmol/l genistein)				
	G_{leak} (μS/cm^2)	G_{Cl}[a] (μS/cm^2)	G_{Br}/G_{Cl}	G_{NO_3}/G_{Cl}	G_I/G_{Cl}
Mean ± S.E.	351 ± 69	1255 ± 347	0.70 ± 0.09	0.53 ± 0.14	0.18 ± 0.05
N	5	5	5	5	5

(conf. Fig. 4 and legend of Table 3).

[a] This is the quasi-stationary conductance. The peak Cl^- conductance averaged 1605 ± 342 μS/cm^2 ($N=5$).

[30]. Finally, we will show (Table 3) that the activated conductance is not affected by adding furosemide to the serosal solution at a concentration which is known to block subepidermal gland secretion.

Binding of the agonist to the β-adrenergic receptor appears to be governed by second-order reaction kinetics as revealed by a sigmoid relationship between the logarithm of the isoproterenol concentration ($\log_{10}$[iso]) and the relative conductance activation with a Hill-slope near unity (Fig. 2). The full curve in this figure is the best fit of Eq. (1), in which the conductance at a given ligand concentration is expressed as a fraction of the fully stimulated conductance, and the baseline conductance recorded prior to addition of the agonist set to zero:

$$\text{Fractional stimulation} = \frac{1}{1+\left(\frac{EC_{50}}{[iso]}\right)^{p}} \quad (1)$$

Here [iso] is the concentration in mol/l, p the Hill coefficient, and EC_{50} the agonist concentration at which half of the full response is recorded. For seven preparations, we obtained $\log_{10}EC_{50} = -7.1 \pm 0.2$ and $p = 1.1 \pm 0.2$ (mean ± S.E., $N=7$).

3.2. Selectivity of the receptor-activated conductance of the intact epithelium

The receptor-activated conductance was dependent on the anion in the external perfusion solution (Fig. 3A). Serosal application of noradrenalin resulted in a conductance activation of similar selectivity (Fig. 2B). In some preparations, the activated conductance passed through a peak value for partially decaying during the subsequent period of several minutes. Examples of this type of 'desensitization' are given in Figs. 1 and 3B. In other preparations, a slow monotonous development of the conductance activation was recorded as illustrated in Fig. 3A. These types of responses were recorded both with isoproterenol and noradrenalin. With the conductance of the epithelium perfused on the outside with gluconate–Ringer's solution taken as the conductance of the 'leak' of the preparation (G_{leak}), the relative conductance sequence of the receptor-controlled pathway (G_A/G_{Cl}) can be estimated from the following expression:

$$\frac{G_A}{G_{Cl}} = \frac{G'_A - G_{leak}}{G'_{Cl} - G_{leak}} \quad (2)$$

Here, $G'_{Cl}$is the conductance recorded in the presence of Cl^- immediately prior to shift to another external anion (A) and $G'_A$the conductance recorded during exposure to A. Calculated in this way, we could take into account that some preparations exhibited partial 'desensitization' as discussed above. The results are compiled in Table 3, which indicate similar anion-selectivity sequence with isoproterenol and noradrenalin, $G_{Cl} > G_{Br} \geq G_{NO_3} > G_I$. Thus, all four anions tested permeate the receptor-controlled apical chloride channel. In the majority of experiments, the conductance was determined by applying a voltage pulse every 20 s across the epithelium of 2-s duration and 20 mV amplitude (serosal side grounded, see Fig. 1). In the experiments denoted 3-iso of Table 3, the pulse polarity was reversed. The results indicate that the conductance sequence of the four anions tested is independent of the direction of the transepithelial voltage step.

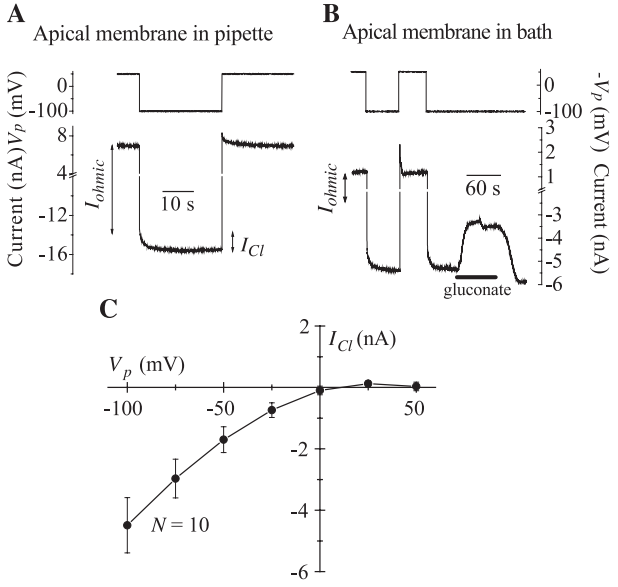


Fig. 6. Recordings from isolated mitochondria-rich cells. (A) With the neck of the cell positioned in the tip of a low resistance patch clamp electrode (about 1 MΩ) and bath connected to ground, the pipette potential, V_p, is a measure of the transcellular potential difference. The current responding to a shift of V_p from +50 to −100 mV is composed of an instantaneous ohmic component (I_{ohmic}) and a more slowly developing dynamic component (I_{Cl}) with the latter representing activation of the apical Cl^- conductance [26]. (B) An experiment with a similar protocol but with the apical membrane of the mitochondria-rich cell exposed to bath. The activation of I_{Cl} was fully reproducible and brief exposure to a Cl^--free gluconate solution eliminated the non-linear component verifying that it is carried by a flow of chloride ions from bath to pipette. (C) Apical membrane facing pipette solution. The steady state I_{Cl} is strongly rectifying illustrating a large flow of chloride ions in the direction from pipette to bath for $V_p < 0$ mV (i.e., the physiological range of transepithelial potential differences). Mean ± S.E. of $N=10$ cells.

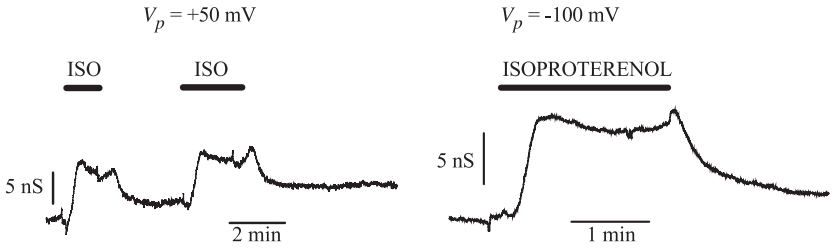


Fig. 7. Demonstration of functional β-adrenergic receptors on the basolateral membrane of mitochondria-rich cells. Left hand panel. Brief superfusion of the mitochondria-rich cell with a Ringer's solution containing isoproterenol (black bars) resulted in reversible and reproducible transcellular conductance activation. Right hand panel. Also in cells clamped at − 100 mV was the transcellular conductance activated by the β-adrenergic receptor agonist.

3.3. Pharmacology of the receptor-activated conductance of the intact epithelium

Heterologously expressed human CFTR-mediated whole cell currents are reduced by the antidiabetic sulfonylurea compound, glibenclamide [31]. In our preparation of an anuran epithelium, the addition of this inhibitor at a concentration of 200 μmol/l in the serosal solution resulted in a significant depression of the isoproterenol-activated transepithelial conductance (Figs. 4 and 5). The isoflavone genistein is a potent kinase inhibitor, which was shown to enhance the cyclic AMP-activated CFTR-mediated Cl^- secretion in a mammalian cell culture [32,33]. The results given in Figs. 4 and 5 show that 50 μmol/l genistein added post-isoproterenol stimulated the transepithelial conductance to a value that is significantly above that of the receptor-activated conductance. By comparing results listed in Tables 3 and 4, it can be seen that the anion conductance sequence of isoproterenol + genistein-stimulated preparations is similar to that of preparations stimulated by isoproterenol only.

3.4. Studies on single mitochondria-rich cells

For investigating the cellular locus of the receptor-controlled Cl^- conductance, we extended the analysis to single cells. The majority of mitochondria-rich cells has a flask-like or slender cylindrical shape, which is maintained following isolation while principal cells become spherical [25]. With low-resistance patch clamp glass electrodes and a small negative pressure, the 'neck' of a mitochondria-rich cell can be manipulated into the tip of the electrode. In this configuration, cell currents can be studied by transcellular voltage clamping [26]. Fig. 6A shows that the voltage clamp current can be resolved in a linear (ohmic) and a non-linear (time-dependent) component. In the previous study, the evidence was presented that the latter is carried by Cl^- [26]. This hypothesis shall be verified below. Accordingly, the non-linear current is denoted, I_{Cl}. In the example shown in Fig. 6A, a shift of the transcellular electrical potential difference (V_p) from + 50 to − 100 mV resulted in a chloride current of $I_{Cl} = -2.3$ nA. In two experiments, we were able to position a mitochondria-rich cell with the apical membrane in the bath. Similar results were obtained with these two cells and results from one of the cells are shown in Fig. 6B. Like in Fig. 6A, the transcellular conductance was activated at − 100 mV from a holding potential of 50 mV (signs referring to apical side). For this cell, $I_{Cl} = -3.4$ nA. Notably, brief exposure of the apical plasma membrane to a Cl^--free solution (gluconate substitution, Fig. 6B) reversibly eliminated the non-linear component, which provides direct evidence that it is carried predominantly by a flux of Cl^- from the apical to the basolateral side of the mitochondria-rich cell. In the present study, at − 100 mV, the Cl^- currents ranged from − 1.2 to − 8.5 nA/cell ($I_{Cl} = -4.5 \pm 0.9$ nA/cell, mean ± S.E., $N = 10$, $V_p = -100$ mV), which is within the range of currents reported in our previous study of 31 mitochondria-rich cells [26]. The voltage dependence of I_{Cl} obtained in the present study of cells from animals kept in distilled water (Section 2) are summarized in Fig. 6C. The chloride currents exhibit a strongly outwardly rectifying I_{Cl}/V relationship with vanishing currents for $V \geq 25$ mV. A similar relationship characterizes the Cl^- currents of the intact epithelium (recently reviewed in Ref.

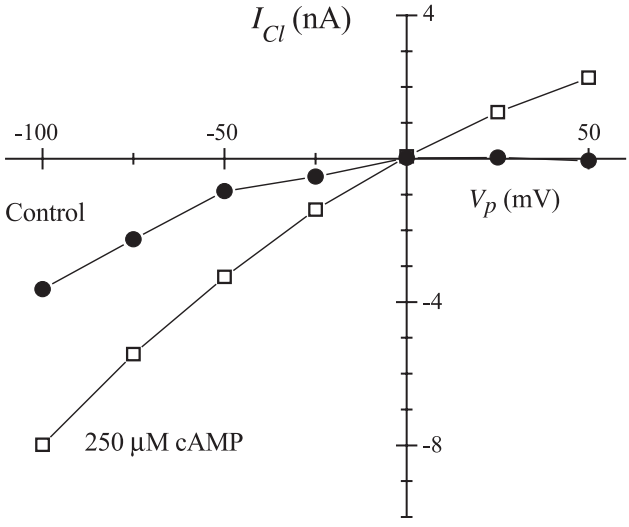


Fig. 8. db-cyclic AMP (250 μmol/l) in bath stimulated the chloride currents at all pipette potentials (− 100 mV ≤ V_p ≤ 50 mV). The I_{Cl}/V relationships were recorded from the same cell before and after the addition of cyclic AMP to bath. It was assumed that the leakage conductance estimated prior to cyclic AMP treatment was not affected by the stimulation of the cellular conductance.

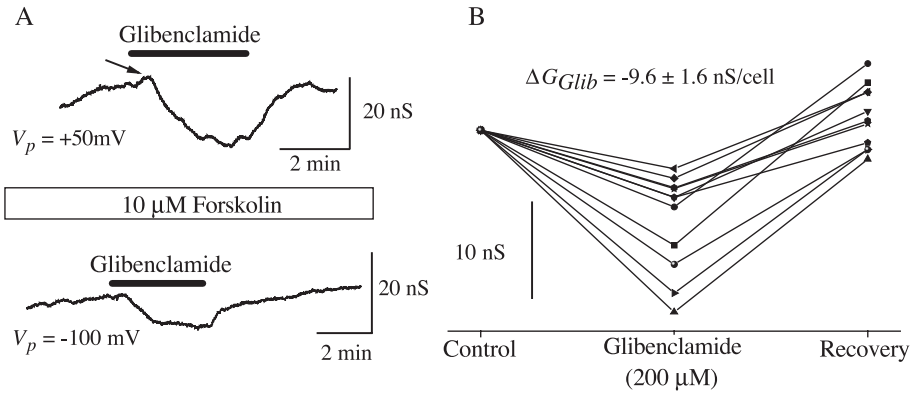


Fig. 9. (A) Examples of time course of glibenclamide-induced reversible conductance inhibition of single mitochondria-rich cells. The cells were exposed to 10 μmol/l forskolin throughout, and during the period indicated by the horizontal bar, 200 μmol/l glibenclamide was present in the forskolin containing perfusate. The two experiments shown were performed with the cell clamped at V_p = 50 mV and − 100 mV, respectively. The recording shown in the upper panel indicates conductance activation prior to glibenclamide inhibition (arrow). (B) Glibenclamide caused a conductance decrease of, ΔG_{Glib} = − 9.6 ± 1.6 nS (n = 11, N = 6 cells). Following superfusion with glibenclamide-free Ringer's solution, the conductance returned to a value that was 1.4 ± 1.0 nS above the conductance recorded prior to adding glibenclamide.

[24]) and of single cells isolated from toads in ion balance [26].

With Cl^- currents deactivated at V_p = + 50 mV, isoproterenol stimulated the transcellular conductance (Fig. 7, left hand panel). A similar response was seen in cells clamped at − 100 mV (Fig. 7, right hand panel). In one cell, the response to different agonist concentrations, 10, 50, and 100 nM, was studied. With these three different concentrations, quantitatively similar effects were obtained, ΔG_{Iso} = 7.2, 7.7, and 7.0 nS/cell, respectively (V_p = + 50 mV). This indicates that 50 nM isoproterenol represents a saturating concentration. In 16 experiments with five cells, the isoproterenol-induced conductance, ΔG_{Iso}, ranged from 7 to 29 nS/cell with an average of 11.9 ± 1.6 nS/cell (mean ± S.E., n = 16). Addition of the membrane-permeable form of cyclic AMP to the bathing solution also stimulated the chloride currents at all potentials. This is shown in Fig. 8 depicting I_{Cl}/V relationships for a cell before and after cyclic AMP treatment.

The cyclic AMP-activated currents were inhibited by glibenclamide at both negative and positive pipette potentials. As shown in Fig. 9A, in cells pretreated with 10 μmol/l forskolin, brief exposure of 200 μmol/l glibenclamide reversibly reduced the transcellular conductance. In some cells, a small conductance stimulation preceded the conductance inhibition (arrow Fig. 9A). Results of 11 experiments with six cells are summarized in Fig. 9B, showing that, on average, glibenclamide reduced the transcellular conductance by 10 nS, which was recovered following wash with glibenclamide-free saline. Thus, glibenclamide is a reversible inhibitor of the targeted ion channel.

3.5. Toad skin CFTR

By RT-PCR we generated full-length cDNA of *bb*CFTR by homology cloning using three pairs of sequence-specific primers each spanning about 1500 bp of the cloned *x*CFTR cDNA. The three bands of the reverse-transcribed and amplified messages (Fig. 10) were ligated into a vector for manual sequencing. The derived amino acid sequence is given in Fig. 11. The *bb*CFTR encodes a 1466 amino acid protein with homology at protein level of 86% with *x*CFTR and 89% of *h*CFTR, respectively. With residue numbers referring to the *bb*CFTR protein, both the R-domain (588–864) and the NBF1 (434–587) and NBF2 (1227–1394) domains are well-conserved. The predicted transmembrane segments (underscored in Fig. 11) indicate conserved amino

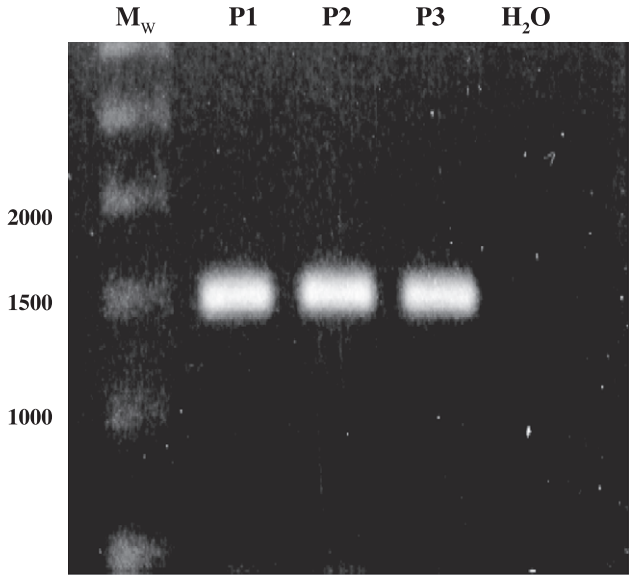
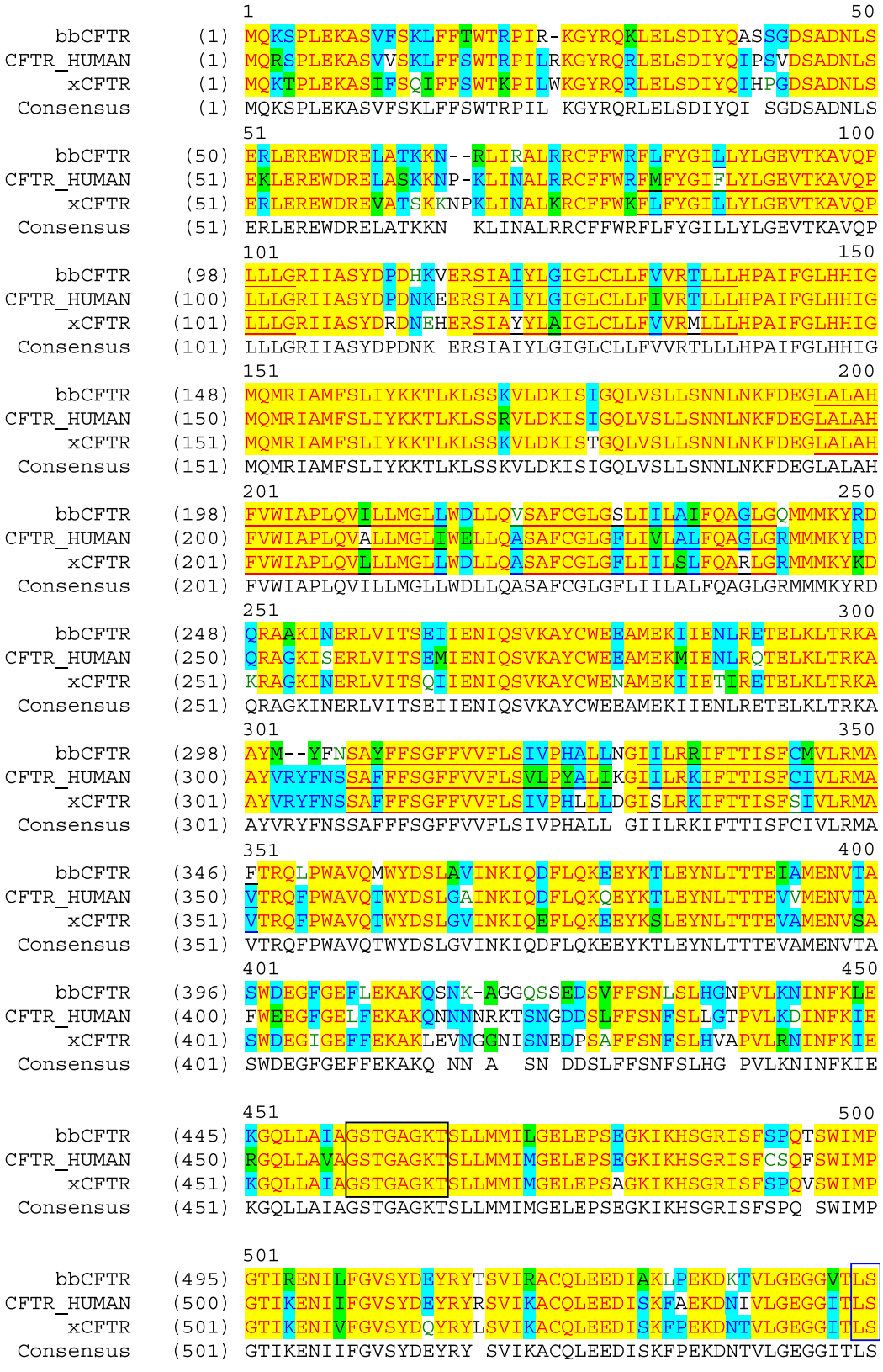
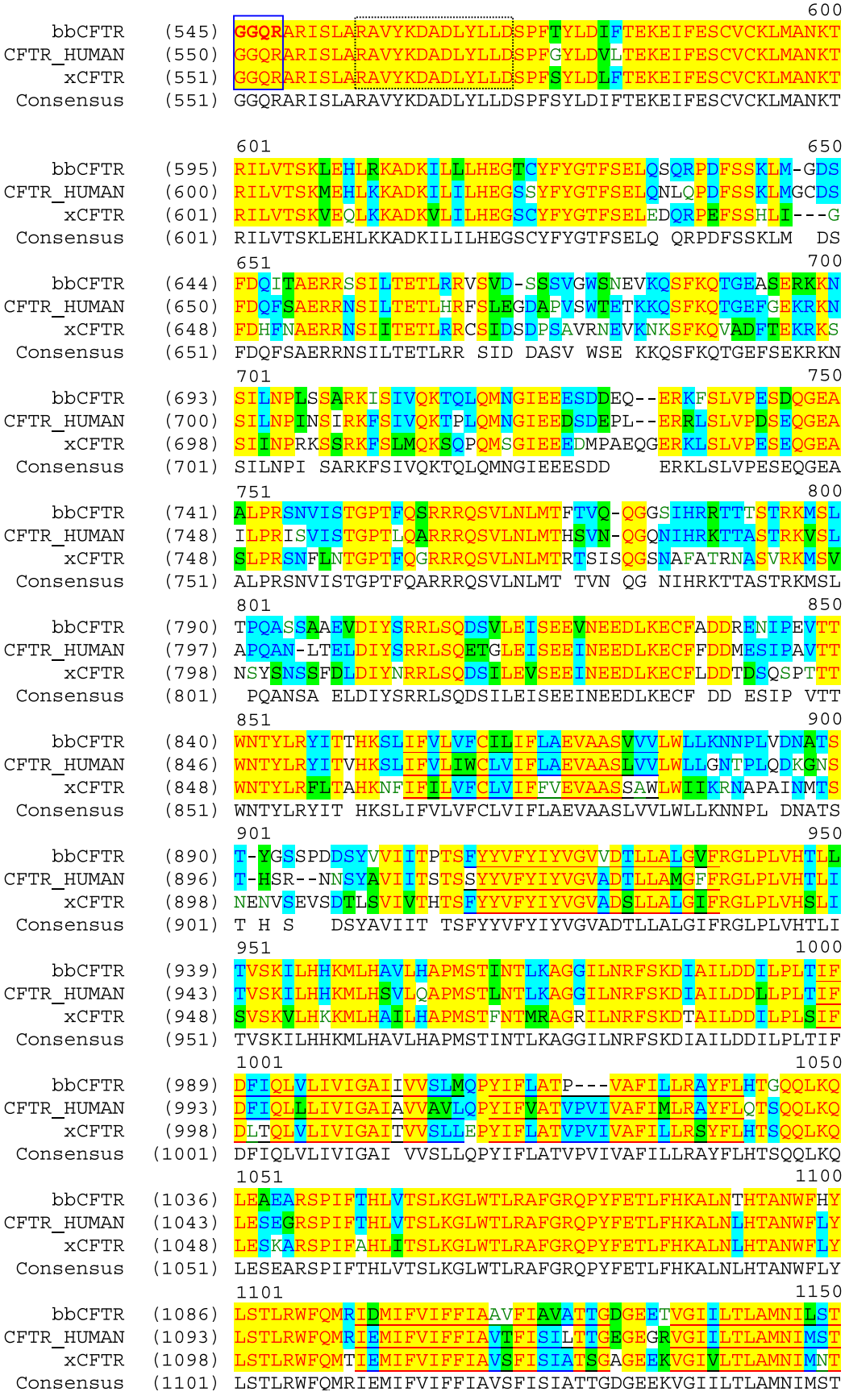


Fig. 10. *bb*CFTR expression in toad skin. Total RNA from toad skin was reverse-transcribed, and the resulting cDNA was PCR-amplified using primers spanning *Xenopus* CFTR (see Section 2). The three bands correspond to the 5′, middle, and 3′ end of *bb*CFTR, respectively.


1 50
bbCFTR (1) MQKSPLEKASVFSKLFFTWTRPIR-KGYRQKLELSDIYQASSGDSADNLS
CFTR_HUMAN (1) MQRSPLEKASVVSKLFFSWTRPILRKGYRQRLELSDIYQIPSVDSADNLS
xCFTR (1) MQKTPLEKASIFSQIFFSWTKPILWKGYRQRLELSDIYQIHPGDSADNLS
Consensus (1) MQKSPLEKASVFSKLFFSWTRPIL KGYRQRLELSDIYQI SGDSADNLS
51 100
bbCFTR (50) ERLEREWDRELATKKN--RLIRALRRCFFWRFLFYGILLYLGEVTKAVQP
CFTR_HUMAN (51) EKLEREWDRELASKKNP-KLINALRRCFFWRFMFYGIFLYLGEVTKAVQP
xCFTR (51) ERLEREWDREVATSKKNPKLINALKRCFFWKFLFYGILLYLGEVTKAVQP
Consensus (51) ERLEREWDRELATKKN KLINALRRCFFWRFLFYGILLYLGEVTKAVQP
101 150
bbCFTR (98) LLLGRIIASYDPDHKVERSIAIYLGIGLCLLFVVRTLLLHPAIFGLHHIG
CFTR_HUMAN (100) LLLGRIIASYDPDNKEERSIAIYLGIGLCLLFIVRTLLLHPAIFGLHHIG
xCFTR (101) LLLGRIIASYDRDNEHERSIAYYLAIGLCLLFVVRMLLLHPAIFGLHHIG
Consensus (101) LLLGRIIASYDPDNK ERSIAIYLGIGLCLLFVVRTLLLHPAIFGLHHIG
151 200
bbCFTR (148) MQMRIAMFSLIYKKTLKLSSKVLDKISIGQLVSLLSNNLNKFDEGLALAH
CFTR_HUMAN (150) MQMRIAMFSLIYKKTLKLSSRVLDKISIGQLVSLLSNNLNKFDEGLALAH
xCFTR (151) MQMRIAMFSLIYKKTLKLSSKVLDKISTGQLVSLLSNNLNKFDEGLALAH
Consensus (151) MQMRIAMFSLIYKKTLKLSSKVLDKISIGQLVSLLSNNLNKFDEGLALAH
201 250
bbCFTR (198) FVWIAPLQVILLMGLLWDLLQVSAFCGLGSLIILAIFQAGLGQMMMKYRD
CFTR_HUMAN (200) FVWIAPLQVALLMGLIWELLQASAFCGLGFLIVLALFQAGLGRMMMKYRD
xCFTR (201) FVWIAPLQVLLLMGLLWDLLQASAFCGLGFLIILSLFQARLGRMMMKYKD
Consensus (201) FVWIAPLQVILLMGLLWDLLQASAFCGLGFLIILALFQAGLGRMMMKYRD
251 300
bbCFTR (248) QRAAKINERLVITSEIIENIQSVKAYCWEEAMEKIIENLRETELKLTRKA
CFTR_HUMAN (250) QRAGKISERLVITSEMIENIQSVKAYCWEEAMEKMIENLRQTELKLTRKA
xCFTR (251) KRAGKINERLVITSQIIENIQSVKAYCWENAMEKIIETIRETELKLTRKA
Consensus (251) QRAGKINERLVITSEIIENIQSVKAYCWEEAMEKIIENLRETELKLTRKA
301 350
bbCFTR (298) AYM--YFNSAYFFSGFFVVFLSIVPHALLNGIILRRIFTTISFCMVLRMA
CFTR_HUMAN (300) AYVRYFNSSAFFFSGFFVVFLSVLPYALIKGIILRKIFTTISFCIVLRMA
xCFTR (301) AYVRYFNSSAFFFSGFFVVFLSIVPHLLLDGISLRKIFTTISFSIVLRMA
Consensus (301) AYVRYFNSSAFFFSGFFVVFLSIVPHALL GIILRKIFTTISFCIVLRMA
351 400
bbCFTR (346) FTRQLPWAVQMWYDSLAVINKIQDFLQKEEYKTLEYNLTTTEIAMENVTA
CFTR_HUMAN (350) VTRQFPWAVQTWYDSLGAINKIQDFLQKQEYKTLEYNLTTTEVVMENVTA
xCFTR (351) VTRQFPWAVQTWYDSLGVINKIQEFLQKEEYKSLEYNLTTTEVAMENVSA
Consensus (351) VTRQFPWAVQTWYDSLGVINKIQDFLQKEEYKTLEYNLTTTEVAMENVTA
401 450
bbCFTR (396) SWDEGFGEFLEKAKQSNK-AGGQSSEDSVFFSNLSLHGNPVLKNINFKLE
CFTR_HUMAN (400) FWEEGFGELFEKAKQNNNNRKTSNGDDSLFFSNFSLLGTPVLKDINFKIE
xCFTR (401) SWDEGIGEFFEKAKLEVNGGNISNEDPSAFFSNFSLHVAPVLRNINFKIE
Consensus (401) SWDEGFGEFFEKAKQ NN A SN DDSLFFSNFSLHG PVLKNINFKIE
451 500
bbCFTR (445) KGQLLAIAGSTGAGKTSLLMMILGELEPSEGKIKHSGRISFSPQTSWIMP
CFTR_HUMAN (450) RGQLLAVAGSTGAGKTSLLMMIMGELEPSEGKIKHSGRISFCSQFSWIMP
xCFTR (451) KGQLLAIAGSTGAGKTSLLMMIMGELEPSAGKIKHSGRISFSPQVSWIMP
Consensus (451) KGQLLAIAGSTGAGKTSLLMMIMGELEPSEGKIKHSGRISFSPQ SWIMP
501
bbCFTR (495) GTIRENILFGVSYDEYRYTSVIRACQLEEDIAKLPEKDKTVLGEGGVTLS
CFTR_HUMAN (500) GTIKENIIFGVSYDEYRYRSVIKACQLEEDISKFAEKDNIVLGEGGITLS
xCFTR (501) GTIKENIVFGVSYDQYRYLSVIKACQLEEDISKFPEKDNTVLGEGGITLS
Consensus (501) GTIKENIIFGVSYDEYRY SVIKACQLEEDISKFPEKDNTVLGEGGITLS


600
bbCFTR (545) GGQRARISLARAVYKDADLYLLDSPFTYLDIFTEKEIFESCVCKLMANKT
CFTR_HUMAN (550) GGQRARISLARAVYKDADLYLLDSPFGYLDVLTEKEIFESCVCKLMANKT
xCFTR (551) GGQRARISLARAVYKDADLYLLDSPFSYLDLFTEKEIFESCVCKLMANKT
Consensus (551) GGQRARISLARAVYKDADLYLLDSPFSYLDIFTEKEIFESCVCKLMANKT
601 650
bbCFTR (595) RILVTSKLEHLRKADKILLLHEGTCYFYGTFSELQSQRPDFSSKLM-GDS
CFTR_HUMAN (600) RILVTSKMEHLKKADKILILHEGSSYFYGTFSELQNLQPDFSSKLMGCDS
xCFTR (601) RILVTSKVEQLKKADKVLILHEGSCYFYGTFSELEDQRPEFSSHLI---G
Consensus (601) RILVTSKLEHLKKADKILILHEGSCYFYGTFSELQ QRPDFSSKLM DS
651 700
bbCFTR (644) FDQITAERRSSILTETLRRVSVD-SSSVGWSNEVKQSFKQTGEASERKKN
CFTR_HUMAN (650) FDQFSAERRNSILTETLHRFSLEGDAPVSWTETKKQSFKQTGEFGEKRKN
xCFTR (648) FDHFNAERRNSITETLRRCSIDSDPSAVRNEVKNKSFKQVADFTEKRKS
Consensus (651) FDQFSAERRNSILTETLRR SID DASV WSE KKQSFKQTGEFSEKRKN
701 750
bbCFTR (693) SILNPLSSARKISIVQKTQLQMNGIEEESDDEQ--ERKFSLVPESDQGEA
CFTR_HUMAN (700) SILNPINSIRKFSIVQKTPLQMNGIEEDSDEPL--ERRLSLVPDSEQGEA
xCFTR (698) SIINPRKSSRKFSLMQKSQPQMSGIEEEDMPAEQGERKLSLVPESEQGEA
Consensus (701) SILNPI SARKFSIVQKTQLQMNGIEEESDD ERKLSLVPESEQGEA
751 800
bbCFTR (741) ALPRSNVISTGPTFQSRRRQSVLNLMTFTVQ-QGGSIHRRTTISTRKMSL
CFTR_HUMAN (748) ILPRISVISTGPTLQARRRQSVLNLMTHSVN-QGQNIHRKTTASTRKVSL
xCFTR (748) SLPRSNFLNTGPTFQGRRRQSVLNLMTRISISQGSNAFATRNASVRKMSV
Consensus (751) ALPRSNVISTGPTFQARRRQSVLNLMT TVN QG NIHRKTTASTRKMSL
801 850
bbCFTR (790) TPQASSAAEVDIYSRRLSQDSVLEISEEVNEEDLKECFADDRENIPEVTT
CFTR_HUMAN (797) APQAN-LTELDIYSRRLSQETGLEISEEINEEDLKECFFDDMESIPAVTT
xCFTR (798) NSYSNSSFDLDIYNRRLSQDSILEVSEEINEEDLKECFLDDTDSQSPTTT
Consensus (801) PQANSA ELDIYSRRLSQDSILEISEEINEEDLKECF DD ESIP VTT
851 900
bbCFTR (840) WNTYLRYITTHKSLIFVLVFCILIFLAEVAASVVVLWLLKNNPLVDNATS
CFTR_HUMAN (846) WNTYLRYITVHKSLIFVLIWCLVIFLAEVAASLVVLWLLGNTPLQDKGNS
xCFTR (848) WNTYLRFLTAHKNFIFILVFCLVIFFVEVAASSAWLWIIKRNAPAINMTS
Consensus (851) WNTYLRYIT HKSLIFVLVFCLVIFLAEVAASLVVLWLLKNNPL DNATS
901 950
bbCFTR (890) T-YGSSPDDSYVVIITPTSFYYVFYIYVGVVDTLLALGVFRGLPLVHTLL
CFTR_HUMAN (896) T-HSR--NNSYAVIITSTSSYYVFYIYVGVADTLLAMGFFRGLPLVHTLI
xCFTR (898) NENVSEVSDTLSVIVTHTSFYYVFYIYVGVADSLLALGIFRGLPLVHSLI
Consensus (901) T H S DSYAVIIT TSFYYVFYIYVGVADTLLALGIFRGLPLVHTLI
951 1000
bbCFTR (939) TVSKILHHKMLHAVLHAPMSTINTLKAGGILNRFSKDIAILDDILPLTIF
CFTR_HUMAN (943) TVSKILHHKMLHSVLQAPMSTLNTLKAGGILNRFSKDIAILDDLLPLTIF
xCFTR (948) SVSKVLHKKMLHAILHAPMSTFNTMRAGRILNRFSKDTAILDDILPLSIF
Consensus (951) TVSKILHHKMLHAVLHAPMSTINTLKAGGILNRFSKDIAILDDILPLTIF
1001 1050
bbCFTR (989) DFIQLVLIVIGAIIVVSLMQPYIFLATP---VAFILLRAYFLHTGQQLKQ
CFTR_HUMAN (993) DFIQLLLIVIGAIAVVAVLQPYIFVATVPVIVAFIMLRAYFLQTSQQLKQ
xCFTR (998) DLTQLVLIVIGAITVVSLLEPYIFLATVPVIVAFILLRSYFLHTSQQLKQ
Consensus (1001) DFIQLVLIVIGAI VVSLLQPYIFLATVPVIVAFILLRAYFLHTSQQLKQ
1051 1100
bbCFTR (1036) LEAEARSPIFTHLVTSLKGLWTLRAFGRQPYFETLFHKALNTHTANWFHY
CFTR_HUMAN (1043) LESEGRSPIFTHLVTSLKGLWTLRAFGRQPYFETLFHKALNLHTANWFLY
xCFTR (1048) LESKARSPIFAHLITSLKGLWTLRAFGRQPYFETLFHKALNLHTANWFLY
Consensus (1051) LESEARSPIFTHLVTSLKGLWTLRAFGRQPYFETLFHKALNLHTANWFLY
1101 1150
bbCFTR (1086) LSTLRWFQMRIDMIFVIFFIAAVFIAVATTGDGEETVGIILTLAMNILST
CFTR_HUMAN (1093) LSTLRWFQMRIEMIFVIFFIAVTFISILTTGEGEGRVGIILTLAMNIMST
xCFTR (1098) LSTLRWFQMTIEMIFVIFFIAVSFISIATSGAGEEKVGIVLTLAMNIMNT
Consensus (1101) LSTLRWFQMRIEMIFVIFFIAVSFISIATTGDGEEKVGIILTLAMNIMST

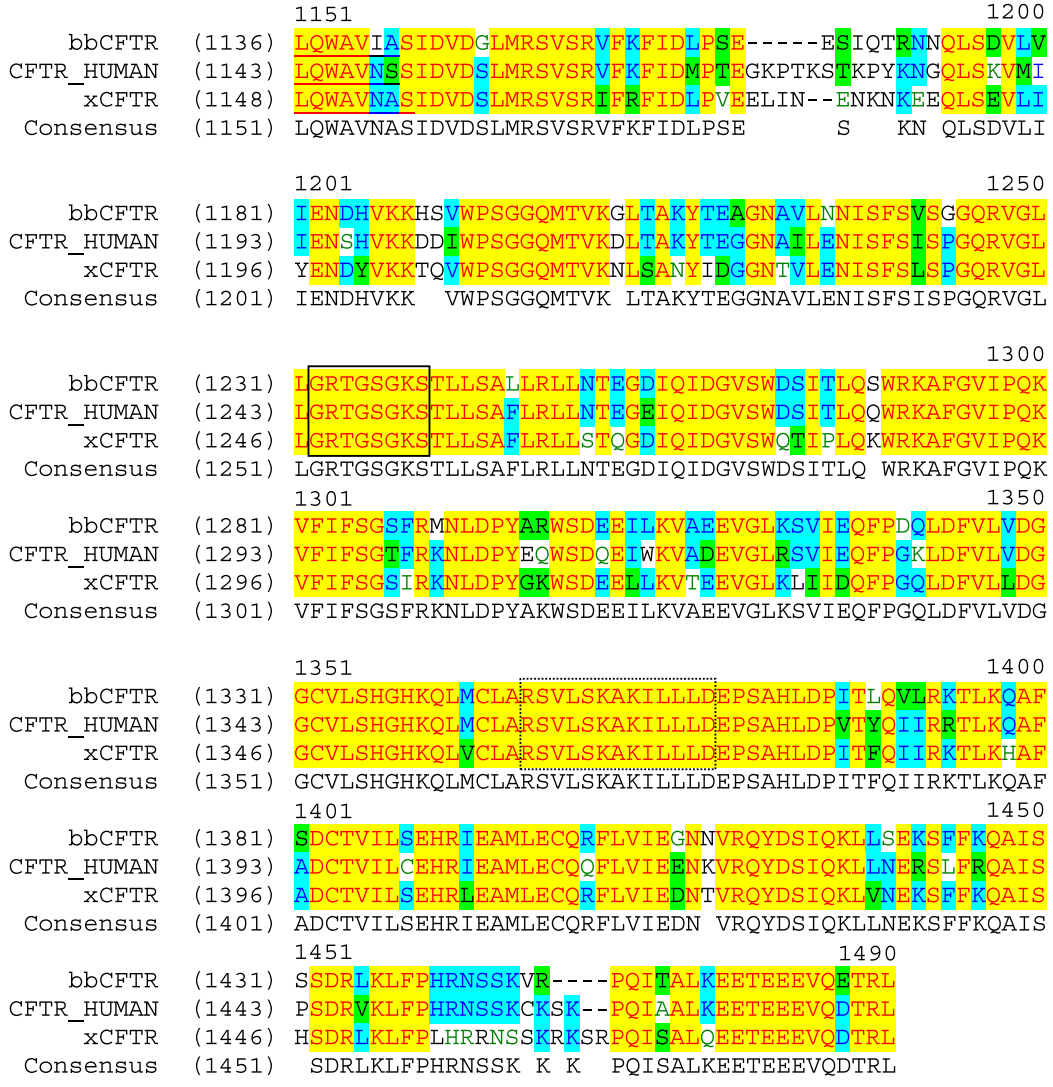


Fig. 11. *bb*CFTR homology alignment. Alignment of the amino acid sequences of *B. bufo* CFTR (*bb*), human CFTR (*h*CFTR) and *Xenopus* (*x*CFTR). Alignment was performed using Vector NTI (blosum62mt2 score matrix). Identical amino acids (aa) are with a red font colour. Non-similar aa are with a black font colour. Conservative aa are boxed in turquoise with blue font colour. Block of similar aa are boxed in green with a black font colour. Weakly similar aa are with dark-green font colour. Putative transmembrane regions are underlined. Boxes drawn with full line indicate Walker A motifs, the box drawn with dashed line indicates the Walker B motif, and the box drawn with blue edge indicates the ABC transporter signature. The residue numbers given above the sequences refer to the consensus sequence. The residue number given in parenthesis prior to each sequence line is the number of the first residue of the line.

acids in regions assumed to form the pore of the channel protein (see Section 4.1).

4. Discussion

4.1. Expression of functional CFTR in the skin epithelium

In a previous study, we obtained the evidence that isoproterenol acts via a forskolin-activated adenylate cyclase and cyclic AMP [22]. For probing the molecular identity of the receptor-activated cyclic AMP-dependent conductance, in the present study of the intact isolated epithelium, we applied three different experimental protocols.

Firstly, the selectivity study (Fig. 3, Table 3) indicated that the population of channels, which is activated by receptor occupation, exhibits an anion conductance sequence comparable to the cloned human CFTR expressed in *Xenopus* oocytes, which conducts chloride, bromide, and iodide as well as nitrate in the following order of efficiency, $G_{Cl}>G_{Br}\geq G_{NO_3}>G_I$ [34–37]. Two cell line studies reported a bromide conductance of human CFTR that is similar to that of chloride [38,39], and in one study, the nitrate conductance was similar to chloride [38]. Thus, there is an unexplained quantitative variation of this feature of the CFTR channel that may be ascribed to different experimental protocols. There is, however, unanimous agreement among all of the reported results, including those of our

own study, that the three halide ions as well as nitrate conducts significant currents through the cyclic AMP-dependent population of channels. Our results agree with those of Nagel and Van Driessche [40], who studied the power density spectrum of forskolin-activated current fluctuations in the skin of *B. viridis*. They concluded that the population of forskolin-activated channels exhibits a voltage-independent ratio of the open/close probability, and conducts chloride as well as nitrate.

Secondly, we investigated the pharmacology of the receptor-activated conductance by using known CFTR modulators. Chloride conductance inhibition by sulfonylureas is generally taken as an indication of CFTR channel-mediated membrane currents [41]. In a concentration-dependent manner, this reversible inhibitor decreases the open state probability of the cloned human CFTR channel when studied in a cell-free system [42]. The open state probability of native anuran CFTR residing in the apical membrane of the subepidermal glands is also reversibly depressed by application of glibenclamide on the cytosolic side of inside–out patches [21]. The finding that glibenclamide significantly reduces the receptor-activated Cl^- conductance of the gland-free preparation (Figs. 4 and 5) provides compelling evidence for functional expression of anuran CFTR in the absorbing epidermal epithelium. The kinase inhibitor genistein enhanced the isoproterenol-activated conductance (Figs. 4 and 5). This is compatible with a modulator, which acts by increasing the open probability of CFTR channels in the plasma membrane. The molecular mechanism of interaction of genistein with CFTR is still subject to discussion [38]. Recent evidence suggests that this isoflavone compound may enhance the single channel open state probability of human CFTR by binding at NBF2 [43]. The present finding that the genistein-activated conductance has a selectivity sequence similar to that of CFTR (Table 4) provides additional evidence for functional expression of CFTR in the apical plasma membrane of the toad skin epithelium.

Our third approach was to apply a molecular biological method for identifying the isoproterenol activated anion channel. Using RT-PCR technique, we identified the message of the toad CFTR gene in the skin (Fig. 10). The primary structure of the protein derived from the cloned cDNA showed a high degree of identity with human CFTR and *Xenopus* CFTR. The functional groups involved in regulation of the opening of the channel, NBF1, NBF2 and the R-domain [1], are all well-conserved. Of special note, the predicted residues of the putative pore region of the human channel protein, which have been hypothesized to confer the discrimination between anions, e.g., R334, F337, T338, and S341 [34,44–47], are also conserved in *bb*CFTR (R330, F333, T334, and S337, respectively, Fig. 11), which is compatible with our selectivity analysis above (Tables 3 and 4). Another residue in this region that has been studied by point mutation is K335 of *h*CFTR, corresponding to R331 of *bb*CFTR (Fig. 11). Mutation of the basic lysine at this position of *h*CFTR expressed in HeLa cells to the acidic residue, glutamic acid, resulted in a sequence selectivity shift from $G_{Cl} \simeq G_{Br} > G_I > G_F$ (wild-type) to $G_{Br} \simeq G_I > GCI > G_F$ (K335E) [38]. Our analysis of the cyclic AMP-activated apical conductance of the toad skin epithelium would suggest that replacement at this position by natural mutation of lysine by another basic residue, arginine (R331 of Fig. 11), does not have a similar pronounced effect on the halide conductance selectivity (Tables 3 and 4).

4.2. Expression of CFTR in the apical membrane of mitochondria-rich cells

In a previous study with double-barreled Cl^--selective microelectrodes, we showed that isoproterenol, as well as exogenously applied cyclic AMP, evoke a Cl^- conductance, which is not located in the principal cells of the epithelium [22]. With this technique applied to another heterocellular epithelium, the collecting duct of rat kidney, Schlatter et al. [48] arrived at a similar conclusion. The studies presented here of single isolated mitochondria-rich cells provide the evidence that the targeted CFTR Cl^- channels are in this cell type. We showed that mitochondria-rich cells express functional β-adrenergic receptors on their basolateral membrane (Fig. 7). Similar to the intact epithelium [22,24], isoproterenol stimulated the current through single cells at both positive and negative transcellular potential differences (Fig. 7). With a receptor-activated conductance of about 12 nS/cell (Section 3.4) and ~ 60,000 mitochondria-rich cells/cm^2 [49] our single cell recordings account for a tissue conductance of ~ 700 μS/cm^2. This number is within the range of receptor-activated G_{Cl} of intact epithelia (600–900 μS/cm^2, Table 3). Furthermore, the cyclic AMP-activated transcellular conductance was inhibited by glibenclamide (Fig. 9), similar to the chloride conductance of the intact epithelium (Figs. 4 and 5). The inhibition was reversible like the glibenclamide inhibition of the native anuran CFTR Cl^- channel in a cell-free system [20].

Following isolation, the cells became more sensitive to isoproterenol (Sections 3.1 and 3.4). We have no explanation for this. One reason might be the delayed exposure to isoproterenol of mitochondria-rich cells in situ. As the ligand would have to diffuse from the serosal bath through the unstirred layer on the serosal side and the labyrinth of intercellular spaces of three to four layers of principal cells, targeted receptors may desensitize before concentration equilibration is reached. Another explanation may be that the exposure of propranolol for several hours (Section 2) increased their sensitivity to the ligand.

In a patch clamp study, our group identified a ~ 7 pS CFTR-like Cl^- channel in cell-attached and inside–out apical membrane patches of mitochondria-rich cells [50]. The frequency of observing this channel almost doubled after adding forskolin to the cell preparation. Furthermore, in an immuno-histochemical study with monoclonal antibodies raised against human CFTR, it was shown that epitopes of the highly conserved regions of the R-domain

and C-terminus are expressed exclusively in mitochondria-rich cells [23,24].

In conclusion, our studies show that the toad's CFTR gene codes for a Cl^- channel in the apical membrane of the mitochondria-rich cells of the heterocellular epithelium.

Acknowledgements

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