

**Effect of internal F^- on activation of Cystic Fibrosis
Transmembrane Conductance (CFTR) regulator by
forskolin**

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Aim:

The aim of this study was to investigate the effect of internal F^- on the activation of the cystic fibrosis transmembrane conductance regulator (CFTR) by forskolin in the whole cell patch clamp configuration. Experiments were conducted with Nanion's fully automated patch clamp device, the Patchliner[©].

Introduction:

The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is a phosphorylation regulated Cl^- channel. In order for the channel to be active it needs to be phosphorylated. Subsequent binding of ATP to intracellular nucleotide binding sites controls the opening and closing of the channel (Zhou *et al.*, 2006).

Our standard internal solutions contain F^- . Regulation of many ion channels does not seem to be affected by the presence of F^- in the intracellular solution, however, that is not true for every ion channel. F^- , for example, has been shown to activate adenylate cyclase (Eckstein *et al.*, 1979). Forskolin – a commonly used CFTR activator – is known to activate CFTR through activation of adenylate cyclase. This leads to phosphorylation (and hence activation) of CFTR by protein kinase A (PKA). It would be interesting to see if F^- can be used as a substitute for forskolin in its role as a CFTR activator.

In this report we summarize some preliminary measurements of CFTR currents in the whole cell patch clamp configuration with the Patchliner[©] to obtain insight into the effects of F^- on CFTR currents.

Material and Methods:

Cells

Baby hamster kidney cells (BHK) expressing *wt* CFTR or untransfected BHK

Cell culture

Cells were cultured in DMEM-F12, 5 % FBS (+500 μ M methotrexate for cells expressing CFTR) in T-25 culture flasks. They were split every second to third day – when 50-100 % confluent. 10-20 % of the harvested cells were then plated in new flasks. Standard Nanion protocols were used to harvest the cells.

For measurements, cells were harvested when 60-90 % confluent (2-3 days after plating), again, according to Nanion's standard protocol.

Whole cell patch clamp recordings

Whole cell patch clamp recordings were conducted according to Nanion's standard procedure with the 2- or 4- channel Patchliner[®]. All solutions used for patch clamp measurements are listed in Table 1a & b. First, the sealing behavior and endogenous currents of untransfected BHK cells were investigated under conditions where Na⁺- and K⁺-currents could be observed (*standard(F)*, *standard external*) and under conditions where both Na⁺- and K⁺-currents are absent (*Na/K-free internal and external*).

CFTR currents were compared under conditions when internal F⁻ (*standard (F)*) was present and under conditions when the F⁻ was replaced by Cl⁻ (*standard (Cl)*). These experiments were conducted with *standard external* solution.

In all experiments cells were kept at a holding potential of -80 mV. Every 5 s a voltage ramp (-100 mV to +100 mV) was applied and the current response to these voltage ramps was recorded.

To compare currents under different conditions, currents at +95 mV were used. They were determined as the mean current flowing in the 95 mV ± 0.5 mV interval. Data are given as mean ± SEM. Data sets were considered as significantly different when a *student's t-test* revealed a p < 0.05.

Table 1a: Internal solutions

| <i>Standard (F⁻)</i> | <i>standard (Cl⁻)</i> | <i>Na⁺/K⁺ -free</i> |
|---------------------------------|----------------------------------|-------------------------------------------|
| 75 mM KCl | 145 mM KCl | 70 mM CsF |
| 10 mM NaCl | 10 mM NaCl | 70 mM CsAsp |
| 70 mM KF | | |
| 2 mM MgCl ₂ | 2 mM MgCl ₂ | 5 mM MgCl ₂ |
| 10 mM EGTA | 10 mM EGTA | 10 mM CsEGTA |
| 10 mM HEPES | 10 mM HEPES | 10 mM HEPES |
| pH 7.2 (KOH) | pH 7.2 (CsOH) | pH 7.4 (CsOH) |

All internal solutions additionally contained 5mM ATP.

Table 1b: *External solutions*

| <i>standard</i> | <i>Na⁺/K⁺-free</i> |
|----------------------------|------------------------------------------|
| 160 mM NaCl | 145 mM TEA Cl |
| 4.5 mM KCl | |
| 1 mM MgCl ₂ | |
| 2 mM CaCl ₂ | 10 mM CaCl ₂ |
| 5 mM D-Glucose Monohydrate | |
| 10 mM HEPES | 10 mM HEPES (Na ⁺ salt) |
| pH 7.4 (NaOH) | pH 7.4 (CsOH) |

Results

Characteristics of untransfected BHK cells

To test how the BHK cell line behaves on the Patchliner[®] and what currents the cells endogenously express we conducted a set of control measurements with untransfected BHK cells. Cells were subjected to the protocol that we used for the investigation of the CFTR currents: cells were automatically sealed and broken into by the predefined Patchliner[®] protocol. Once a stable whole cell configuration was established voltage ramps (-100 mV to +100 mV) were applied every 5 s and current responses recorded.

To observe what type of currents were present in the untransfected cells we used two sets of solutions: (1) *Na/K* - our standard solutions with external high Na⁺ and internal high K⁺, conditions under which Na⁺- and K⁺-currents can be observed; (2) *TEA/Cs* - solutions in which the external Na⁺ is replaced by TEA⁺ and the internal K⁺ by Cs⁺, conditions under which Na⁺- and K⁺-currents are blocked.

The BHK cells sealed in a similar manner to that observed before for many other cell lines on the Patchliner[®] or Port-a-Patch[®]. Seals steadily increased during the sealing procedure. However, these cells had fragile membranes and the whole cell configuration was often established before a giga seal had been reached. Nevertheless, by the time recordings were started, giga seals had formed (*Na/K*: 1.7 GΩ ± 0.7 GΩ, n = 4; *TEA/Cs*: 3.1 GΩ ± 0.8 GΩ, n = 4). Current responses to the initial voltage ramp (-100 mV to +100 mV) of individual cells under the two different conditions (*Na/K* and *TEA/Cs*) are shown in Fig 1A. Initial currents at +95 mV are small (*Na/K*: 139 pA ± 23 pA, n = 4,

TEA/Cs: $325 \text{ pA} \pm 89 \text{ pA}$, $n = 4$). These small currents indicate that, if at all, only very small K^+ -currents are present.

The current response of the cell under conditions where Na^+ - and K^+ -currents can be observed shows a characteristic Na^+ current “hump” (4/4, Fig. 1A). This was not observed in Na^+ -free (*TEA/Cs*) conditions (4/4) supporting the hypothesis that the hump is caused by Na^+ -flux. Running a Na^+ -current voltage protocol (voltage step from a holding potential of -80 mV to potentials between -60 mV and $+60 \text{ mV}$ for 20 ms in increments of 10 mV , and then back to -80 mV) revealed typical Na^+ current responses Fig. 1B ($n = 1$).

To test what effect forskolin has on currents under the different conditions, voltage ramps were applied every 5 s for a 5 min control period, once the whole cell configuration had been established. Then $2 \mu\text{M}$ forskolin was added and currents were observed for another

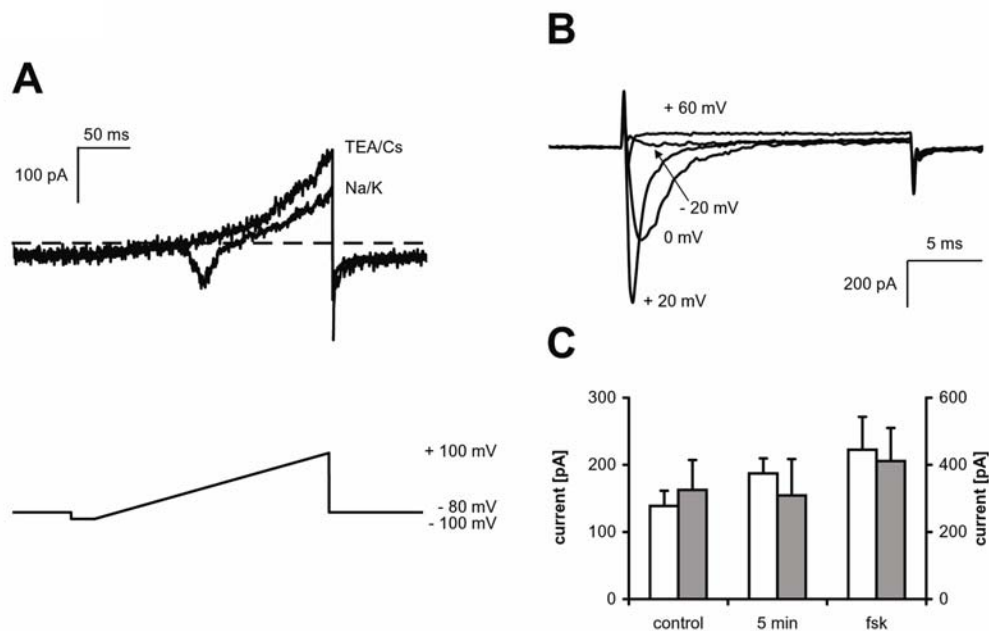


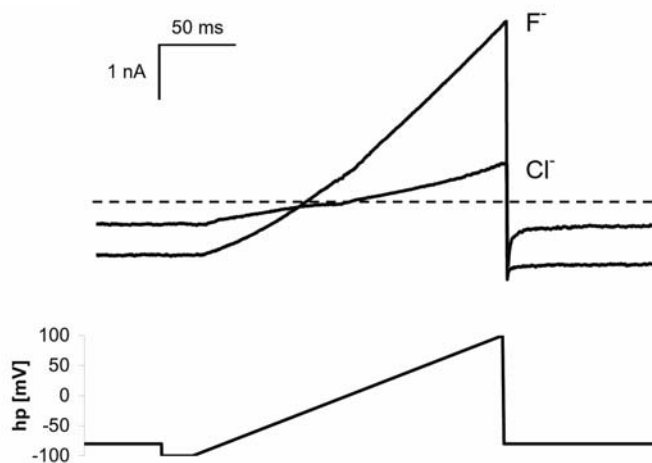
Figure 1: Untransfected BHK cells have Na^+ -currents but no forskolin regulated currents. (A) Current responses (top) to a voltage ramp (bottom) of two individual cells recorded in different conditions (*TEA/Cs*: Na^+ - and K^+ -free solutions, *Na/K* standard solutions internal *standard(F)*, *standard* external). (B) Current response of the cell shown in (A, *Na/K*) to a Na^+ -current pulse protocol. (C) Mean currents at $+95 \text{ mV}$ for cells recorded under *Na/K* conditions (open bars, $n = 4$) and *TEA/Cs* conditions (grey bars, $n = 4$).

5 min before they were evaluated for the effect of forskolin. Currents under neither condition changed significantly during the 5 min control period or within 5 min following exposure to forskolin (Fig. 1C).

Sealing behavior and currents of BHK cells expressing wt CFTR

BHK cells expressing *wt* CFTR were handled according to the same protocols as the untransfected BHK cells. As with the untransfected cells, membranes were fragile and the whole cell configuration was established early in the sealing process, before giga seals were formed. Seals increased steadily as long as the cells remained intact. However, once the whole cell configuration was established, seals of the transfected cells appeared to steadily decline.

Seal resistances, as determined from the current response to a 10mV voltage step from -80mV at the point in time when the first ramp protocol was executed, differed significantly between cells exposed to internal *standard(F⁻)* ($150 \text{ M}\Omega \pm 27 \text{ M}\Omega$, $n = 12$) and *standard(Cl⁻)* ($650 \text{ M}\Omega \pm 257 \text{ M}\Omega$, $n = 3$). This difference in seal resistance is a surprising result since cells recorded with a F^- containing internal solution usually reach at least the same seal resistance as cells recorded in internal solution without F^- , as shown for the *wild-type* BHK. For this reason, it is likely that the lower seal resistances are due to open CFTR channels. CFTR channels are not voltage-activated. The calculation of the seal resistance would drop notably by activation of CFTR channels in the whole cell



*Figure 2: Initial current responses are bigger for cells recorded in the presence of internal F^- than with F^- -free solutions. Shown are current responses (top) to the initial voltage ramp (bottom) for two cells, one recorded with internal *standard(F⁻)* solution (F^-) and one with *standard(Cl⁻)* solution (Cl^-).*

configuration. Fig. 2 shows whole cell currents of BHK cells expressing CFTR, one in the presence of internal Cl^- and one in the presence of internal F^- . This difference in current amplitude can not be seen in *wild-type* BHK (Fig. 1). If this difference is due to activation of CFTR by F^- through the PKA pathway, this effect should not be further enhanced by forskolin.

Fig. 3 shows currents from two individual cells recorded in the presence and absence of internal F^- . Currents in the presence of internal F^- increased with time (Fig. 3A & B) whereas currents recorded in the absence of internal F^- did not change significantly within 5 minutes of recording (Fig. 2A & B). Exposure to 2 μM forskolin 5 min after current recordings in the whole cell configuration had been started did not lead to a significant change in the currents recorded with internal *standard*(F^-) solution. Forskolin activated currents recorded with internal *standard*(Cl^-) (Fig. 3A & B). The reason that the difference is not statistically significant is due to the low n number ($n = 3$), and the variability in the responses. The concentration of 2 μM forskolin is not fully activating (Seamon, Padgett, and Daly, 1981). Interestingly, currents recorded in the presence and

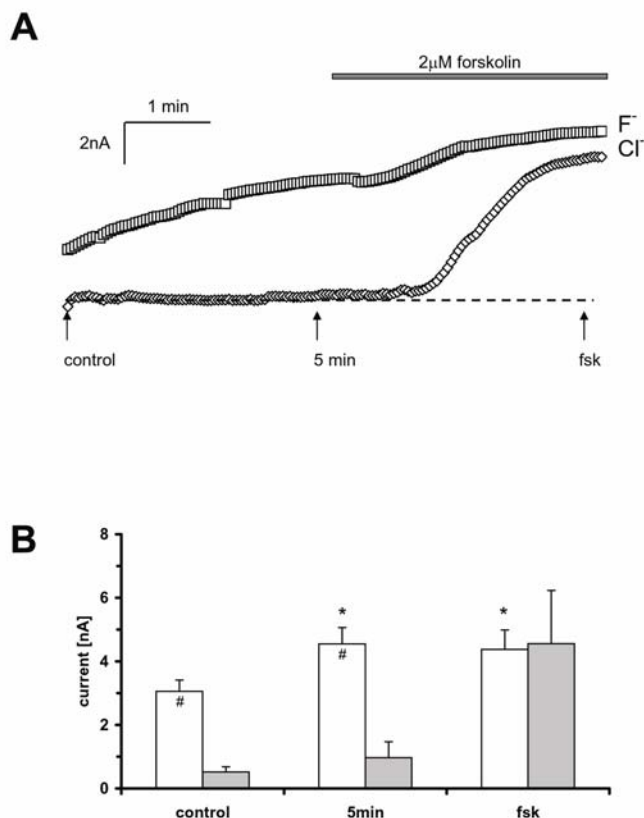


Figure 3: Forskolin does not activate currents further when channels are already activated by F^- . (A) shows the current responses at +95 mV for two individual cells, one recorded with internal *standard*(F^-) solution (F^-) and one with *standard*(Cl^-) solution (Cl^-). (B) Mean currents at +95 mV for cells recorded in the presence of internal F^- (open bars, $n = 12$) and in the absence of internal F^- (grey bars, $n = 3$). * denotes significant difference to data obtained under the same conditions at *control*. # denotes significant difference to data obtained under different conditions (internal F^- vs. Cl^-).

absence of internal F^- did not differ significantly from each other after 5 min exposure to forskolin (Fig. 3B). However, due to the different internal Cl^- concentrations it is not a given that channel activity is the same under both conditions.

Discussion

In this report we summarize some preliminary experiments investigating the effect of intracellular F^- on CFTR currents measured in the whole cell configuration on the Patchliner[®]. This is by no means meant to be a complete study.

In summary, our measurements show that there is an activation of CFTR channels by F^- which cannot be further enhanced by forskolin. Looking at the literature, one explanation for this finding seems very plausible: F^- interferes in the same step as forskolin within the signaling pathway (Fig. 4), although *via* a different mechanism, both F^- and forskolin activate adenylate cyclase (Eckstein *et al.*, 1979, Insel and Ostrom, 2003). Once activated, the cyclase converts ATP into cAMP. This leads to phosphorylation of protein kinase A (PKA) and the kinase becomes active. The active kinase can then phosphorylate the CFTR, openings of which are then controlled by ATP binding to the cytoplasmic nucleotide binding site.

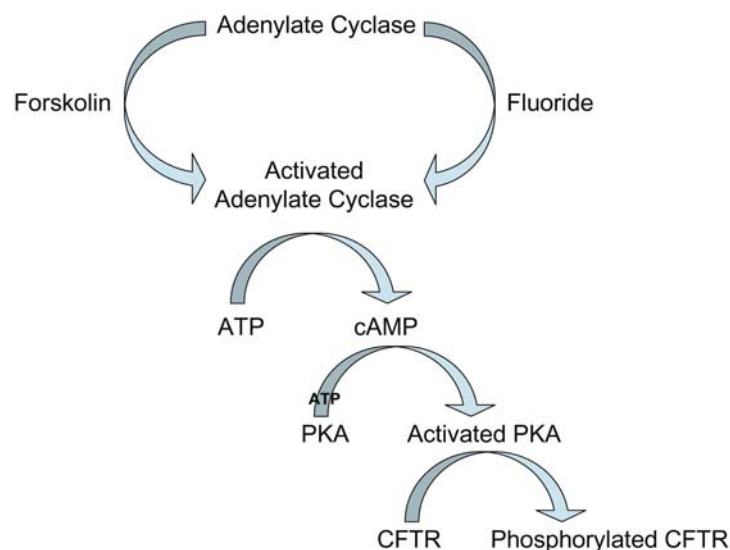


Figure 4: Scheme for the signalling pathway of phosphorylation of CFTR by forskolin and fluoride.

In conclusion, the findings reported in this study suggest that internal F^- activates CFTR by upregulation of adenylate cyclase. This raises the possibility of replacing expensive activator forskolin with internal F^- for CFTR studies. To confirm this will require further investigation.

Literature

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