

CFTR, investigated with the two-electrode voltage clamp technique: consequences of an uncompensated series resistance

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INTRODUCTION

When, in 1989, the gene mutated in Cystic Fibrosis (CF) was cloned by positional cloning and named Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) it was not clear from the sequence what its function might be. Subsequent heterologous expression in different cells (Tabcharani et al., 1991; Anderson et al., 1991) and studies on endogenous CFTR (Nagel et al., 1992) led to the conclusion that CFTR is an anion (or simplified: chloride) channel which is regulated by protein phosphorylation and nucleotides (reviewed in: Riordan, 1993; Gadsby et al., 1995; Gadsby & Nairn 1999; Sheppard & Welsh 1999; Nagel 1999). Most of these electrophysiological studies were patch-clamp studies but CFTR expressed in oocytes of *Xenopus laevis* (Bear et al., 1991; Drumm et al., 1991) was studied in whole oocytes by the two-electrode voltage-clamp (TEVC) technique. This protocol aims to shortly describe TEVC and to highlight the special requirements for studying CFTR with it. Although TEVC is a simple technique that we are also using in a practical course for Biochemistry students, it bears some pitfalls which I would like to draw to the reader's attention. Introductions to TEVC of oocytes may be found in excellent recent reviews (Stühmer 1992; Stühmer 1998; Wagner et al., 2000). In this protocol I will focus on series resistance which often plays no practical role in TEVC of oocytes, e.g. when studying low-turnover transporters. Therefore series resistance is mostly paid little or no attention. As shown below, when studying CFTR or other channels and even more so when studying interaction of CFTR with other channels, series resistance considerations cannot be neglected.

Already in 1981 it was suggested that sodium absorption is increased in CF-airways (Knowles et al. 1983). The epithelial sodium channel (ENaC) was cloned in 1993 and in 1995 appeared the first report of a patch-clamp study suggesting that CFTR inhibits ENaC (Stutts et al., 1995), raising the question whether CFTR has an additional function. Several studies on coexpressed CFTR and ENaC in oocytes have been published since 1996 (Mall et al., 1996; Briel et al., 1998; Chabot et al., 1999, but see Erratum: Chabot et al., 2002!), suggesting that activation of CFTR inhibits ENaC. Intrigued by this possible function of CFTR, we measured CFTR and ENaC currents in excised patches from oocytes coexpressing them, but found no evidence for interaction of CFTR with ENaC (Nagel et al., 2001). When we then also in whole oocytes, at clamped membrane potentials, found no interaction between CFTR and ENaC, we suggested that the previously observed apparent inhibition of ENaC may be explained by a series resistance which was in the range of the oocyte membrane resistance (Nagel et al., 2001). Series Resistance considerations are

often neglected but are important not only in TEVC but also in patch-clamp experiments (also called: Access Resistance), see e.g. the illuminating review by Armstrong and Gilly (1992). Our conclusion was supported by a recently published erratum from the group of Ryszard Grygorczyk (Chabot et al., 2002) and by a meeting abstract (Segal et al., 2002). It is, however, still far from being generally accepted. For example, a recent report by König et al. (2001) concluded that activated CFTR inhibits ENaC without observing a specific interaction between CFTR and ENaC. The apparent inhibition of ENaC (which was also induced by application of the membrane-permeabilizing antibiotic amphotericin B) was suggested to be due to an elevated internal chloride concentration, whereas a series resistance error seemed to be excluded (König et al., 2001). This protocol aims to explain to the interested reader, hopefully even to the non-electrophysiologist, some specific problems in TEVC of CFTR, why series resistance considerations are important, and how they may be dealt with seriously.

Expression of CFTR in oocytes

Expression of membrane proteins in oocytes of *Xenopus laevis* is quite common as it is easy to study electrical currents with Two-Electrode Voltage-Clamping (TEVC). Additionally, oocytes are well suited to express proteins, e.g. from viruses, archaea, algae, plants, and animals (Gurdon et al., 1971; Gundersen et al., 1984; Cao et al., 1992; Nagel et al., 1995; Nagel et al., 2002). The most common CFTR mutant, $\Delta F508$, which virtually does not reach the plasma membrane in mammalian cells, does so in oocytes where its gating may be studied (Drumm et al., 1991).

The oocytes are obtained by surgical removal of a part of the ovary and subsequent digestion with collagenase, as described elsewhere (Grygorczyk et al., 1989; Stühmer, 1992). They are then stored in Ringer's solution (ORi) supplemented with antibiotics. Kits are available nowadays, making it an easy task to prepare mRNA. Usually 10 to 50 nl mRNA solution are injected, corresponding to maximally 50 ng mRNA. Oocytes are then incubated for at least 24 hrs., usually up to five days, in ORi with antibiotics whereby expression increases with time. Only for ENaC we observed a decrease of expression at incubation periods longer than 36 hrs. When expressing ENaC, amiloride (10 μM) has to be added to ORi or the Na^+ concentration has to be reduced (replaced by N-methyl-D-glucamine) to prevent a rapid decline in the viability of oocytes.

Electrodes and electronics

Electrodes and their preparation are explained in another protocol, authored by Martin Hug. In short, for intracellular electrodes glass capillaries are pulled with commercially available pullers to obtain tip openings of a few μm . These electrodes are then filled with 3 M KCl whereby care is taken to avoid air bubbles in the tip. The electrode is then inserted into an electrode holder with a chlorinated silver wire (Ag/AgCl) which enables the change from ionic to electronic current in the electrical circuit. Electrodes are judged depending on their electrical resistance which should be in the range of 1 M Ω .

The term "Two-Electrode Voltage-Clamping" (TEVC) refers to the Cell-impaled electrodes, however we also need at least one reference electrode in the bath

solution, see **fig. 1**. It is advisable to use two bath electrodes, one for returning the current and one for sensing the potential but not every amplifier provides these connections and even if it does, experimenters, for ease, often use only one, combined bath electrode. This common practice is justified as long as oocyte membrane resistance is relatively high, e.g. when expressing low-turnover transporters. Whereas the bath electrode for returning current may be simply an Ag/AgCl-wire, it is advisable to use an "agar bridge" (a capillary filled with 3 M KCl in 2% agar-agar) to connect the Ag/AgCl-bath electrode for sensing membrane potential with the bath solution. In this way the membrane potential measurement is not compromised by changes in chloride concentration. The use of a "flowing 3 M KCl bridge" may further reduce liquid junction potentials (Neher, 1992). TEVC-amplifiers are available from several manufacturers and may be chosen depending on needs and budget. For studies on CFTR, only an amplifier allowing for two bath electrodes should be chosen. As even low amounts of CFTR-mRNA (3 ng) induce a high membrane conductance (up to 0.5 mS), CFTR-mediated current may become large, requiring an amplifier with a high maximal voltage output (≥ 100 V) for injecting current.

Series Resistance considerations in voltage-clamping of oocytes

Figure 1 shows a schematic diagram of TEVC on an oocyte, with two bath electrodes (a) or one bath electrode (b). Simplified, we have an electrical circuit for measuring potential (V) and one for injecting current (I), ideally both involving two electrodes. The membrane resistance is composed of the membrane resistance of endogenous proteins (R_M) and the resistance of expressed proteins (R_X). Although we are only interested to measure the conductance of expressed proteins (the inverse of their resistance, R_X) we have to keep track of the other relevant resistances in the electrical circuit, see **fig. 1a**. As these resistances are in series to the membrane resistance they are called series resistance (R_S). Obviously we have an intracellular series resistance (R_{Si}) between the impaled V-electrode and the membrane and an extracellular series resistance (R_{Se}) between the membrane and the bath V-electrode. The extracellular series resistance is a sum of various components, depending on the experimental conditions, see **fig. 1a + b** and table 1. As the V-circuit includes a high-resistance amplifier input, virtually no current is flowing in the electronic part of this circuit. Current is however flowing in the I-circuit, injected by the impaled Current-electrode and returning via the bath I-electrode, therefore also flowing in the ionic part of the V-circuit, as indicated by arrows in **fig. 1**. According to Ohm's law, a voltage will drop along this pathway where the voltage across each resistance is the product of resistance and electrical current. Relevant resistances are the intracellular series resistance (R_{Si}), the membrane resistance (which we divide into the endogenous membrane resistance, R_M in parallel with the resistance of expressed proteins, R_X), and the extracellular series resistance (R_{Se}), however not the resistances of the V-electrodes as no current is passing through. The intracellular series resistance depends on the shape of the cell and on plasma membrane folding and, fortunately, seems to be low in the case of oocytes (probably less than 100 Ω). The extracellular series resistance in a Two-Bath-Electrodes setup depends only on the distance of the bath V-electrode (i.e. the tip of the "agar bridge" or "flowing KCl bridge") to the oocyte, see **fig. 1a**. If this distance is kept small R_{Se} may be in the range of 100 Ω , see table 1. A larger distance however easily

introduces a series resistance of several k Ω . The extracellular series resistance (R_{Se}) in a One-Bath-Electrode setup not only depends on the distance of the bath V-electrode to the oocyte (R_{SeBa}) but also on the resistance of the "agar bridge" or "flowing KCl bridge" (R_{SeBr}), as well as on the resistance of the Ag/AgCl electrode (R_{SeAg}), see **fig. 1b** and table 1. Below I will describe a simple method to estimate $R_{Se} \approx R_S$. It is important to know R_S for each experiment as it will influence the error in measuring the conductance of expressed proteins.

If, as a rule of thumb, we allow for up to 5% error in estimating R_X then the combined membrane resistance, i.e. $R_X * R_M / (R_X + R_M)$, has to be 2 k Ω or more if $R_S = 100 \Omega$. That means, with $R_S = 100 \Omega$, the measured conductance should not exceed 500 μ S. Or, with $R_S = 1 \text{ k}\Omega$, the measured conductance should not exceed 50 μ S. Table 1 gives estimates of typical values contributing to R_{Se} for setups with one or two bath electrodes. As seen from table 1, with a one-bath-electrode configuration R_{Se} is always higher and it is much harder to fulfill the requirement of a series resistance not exceeding 5% of the overall resistance, especially for channels expressing as well as CFTR and ENaC, i.e. where R_X is small.

Electrical studies on CFTR

CFTR expressed in oocytes may be studied by the patch-clamp method on cell-attached or excised membrane patches or by TEVC of whole oocytes. The advantage of excised patches is the free access to the cytoplasmic side so that modulation of CFTR by phosphorylation and interaction with nucleotides may be studied (Anderson et al, 1991; Tabcharani et al, 1991; Nagel et al., 1992; reviewed in: Gadsby & Nairn, 1999; Sheppard & Welsh, 1999; Nagel, 1999). On the other hand, measuring CFTR activity in whole oocytes has the advantage to include cytoplasmic components and experiments are generally faster and easier, e.g. ideal for rapid screening of mutants. Oocytes express an endogenous Ca-activated chloride channel in large amounts but are free of CFTR-like endogenous channels. To activate CFTR in oocytes the oocyte-endogenous cAMP-dependent protein kinase (PKA) may be used. In fact, even without stimulation, basal [cAMP] leads to a tiny activation of expressed CFTR via PKA (from 2 μ S to 10 μ S, Chan et al., 2000). Stimulation of adenylate cyclase with forskolin (10 μ M) and/or inhibition of phosphodiesterase with iso-butyl-methyl-xanthine (IBMX, 0.5 – 1 mM), however, increases [cAMP], as indicated by the slow rise of a huge chloride-specific conductance in CFTR-expressing oocytes.

A large conductance may be hidden by a series resistance.

CFTR expresses very well in oocytes, i.e. activated CFTR "permeabilizes" oocytes for chloride. Chan et al., 2000, report a basal conductance of 10 μ S for oocytes injected with 2.5 ng CFTR-mRNA which is increased by elevating [cAMP] to 170 μ S. In order to get CFTR-conductances of less than 100 μ S for quantitative analysis of data, Smit et al. (1993) injected as little as 0.1 ng CFTR-mRNA. We obtained a CFTR-mediated conductance of 250 μ S, 2 – 3 days after injecting 3 ng CFTR-mRNA (Nagel et al., 2001). As outlined above, to measure a conductance of 250 μ S reliably it is crucial that the series resistance is 200 Ω or less if the error introduced by the

series resistance should not exceed 5%. Is it possible to measure the series resistance? As seen from the electrical circuit in **fig. 1**, the series resistance ($R_S = R_{Si} + R_{Se}$) is equal to the total resistance if the membrane resistance is decreased to zero! This can be easily achieved, e.g. at the end of an experiment simply by rupturing the oocyte membrane with the impaling electrodes or by pulling the electrodes out of the oocyte, still leaving them close to the oocyte, and measuring conductance (the inverse of total resistance) with small voltage pulses in voltage-clamp mode. Alternatively, the series resistance may be estimated at the beginning of the experiment, before putting an oocyte into the measuring chamber, by placing all electrodes where they would be during voltage-clamping of an oocyte and then switching to voltage clamp with a command potential of 0 mV. The current, flowing to obtain a voltage difference of e.g. 1 mV, is then only determined by the series resistance. For the example of a 1 mV-pulse a current of 10 μ A would indicate a series resistance of 100 Ω , which is acceptable for measuring conductances up to 500 μ S, as outlined above.

Is CFTR a regulator of ENaC?

In giant excised patches with thousands of coexpressed CFTR and ENaC channels we investigated whether activation of CFTR by ATP influences the electrical activity of ENaC. No influence of CFTR activation on ENaC activity was found (Nagel et al., 2001). As it might be possible that a cytoplasmic factor was lost in these excised patches, we studied the effect of CFTR activation on ENaC in whole oocytes with TEVC. But also with TEVC the CFTR conductance and ENaC conductance were purely additive (Nagel et al., 2001). **Fig. 2a** shows results of conductance measurements for an oocyte with a CFTR conductance of 200 μ S, an ENaC conductance of 100 μ S, and a series resistance of 100 Ω . ENaC-conductance (amiloride-sensitive) during CFTR-activation (#6 = #3 - #1) is not significantly different from ENaC-conductance without CFTR-activation (#5 = #2). **Fig. 2b** demonstrates apparent conductances for CFTR and ENaC (for the same oocyte) when measuring with a series resistance of 1 k Ω . Obviously the determined apparent conductances are smaller and the ENaC-conductance during CFTR-activation (#6) appears smaller than the ENaC-conductance when CFTR is inactive (#5). **Fig. 2c,d** show apparent conductances for the same oocyte with even higher series resistances of 5 k Ω and 10 k Ω , respectively. As demonstrated by these examples, increasing the series resistance not only decreases the apparent conductances of CFTR and ENaC, but also suggests a dramatic inhibition of ENaC by activated CFTR. Taken together with the previously reported relatively small conductances of CFTR- and ENaC-expressing oocytes, despite large amounts of injected RNA, we proposed that an uncompensated series resistance could explain the reported "apparent" inhibition of ENaC by activated CFTR (Nagel et al., 2001). This conclusion was recently corroborated by Grygorczyk and coworkers (Chabot et al., 2002, Erratum). After the elimination of a series resistance of 5 to 7 k Ω the previously reported inhibition of ENaC by activated CFTR (Chabot et al., 1999) was no longer observed. Also Segal et al. (2002) found no evidence for inhibition of ENaC by activated CFTR in voltage-clamped oocytes coexpressing CFTR and ENaC.

A final word on regulation by CFTR: Any ion-translocating membrane protein is usually modulated in its activity by membrane potential and, if it is electrogenic, will modulate membrane potential by its activity. Therefore we have to conclude that any

electrogenic ion-transporter (pump, exchanger, symporter, or channel) will indirectly modulate the activity of other ion-transporters if membrane voltage is not clamped. Such an unspecific modulation in non-voltage-clamped cells should not be confused with a specific regulatory relationship between membrane proteins, mediated by protein-protein interaction, for which there are many examples.

We might add that an effect of CFTR activation on amiloride-sensitive sodium uptake in coexpressing oocytes was observed in non-voltage-clamped oocytes. Activation of CFTR at low (2 mM) external $[Cl^-]$ inhibited $^{22}Na^+$ uptake and at high (121 mM) external $[Cl^-]$ stimulated $^{22}Na^+$ uptake, in contrast to the suggestion of König et al. (2001). This unspecific effect was also observed with ClC-0 chloride channels and is easily explained by the consequences of activating a chloride conductance on membrane potential (Nagel et al., 2001). In summary, we conclude that the only modulatory effect of CFTR on ENaC is via the membrane potential and therefore see no reason to search for a direct CFTR-ENaC interaction on the basis of the erroneously reported inhibition. However, because both proteins are needed for effective NaCl absorption, as well documented in the sweat duct (Reddy et al., 1999), they still might be expressed, processed, or regulated in a coordinated fashion.

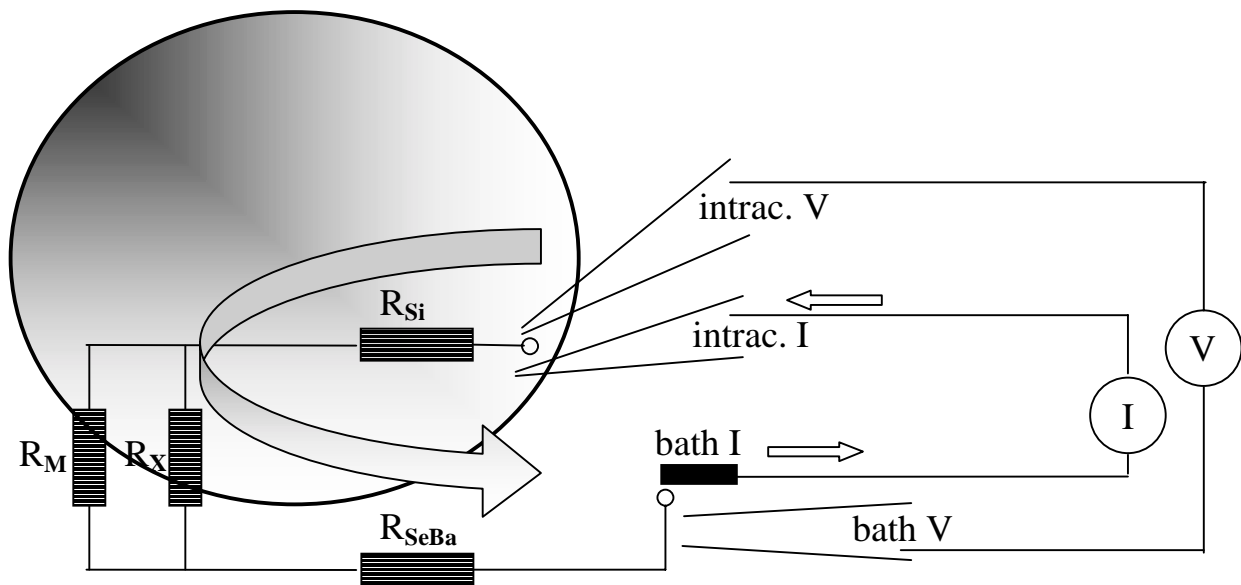
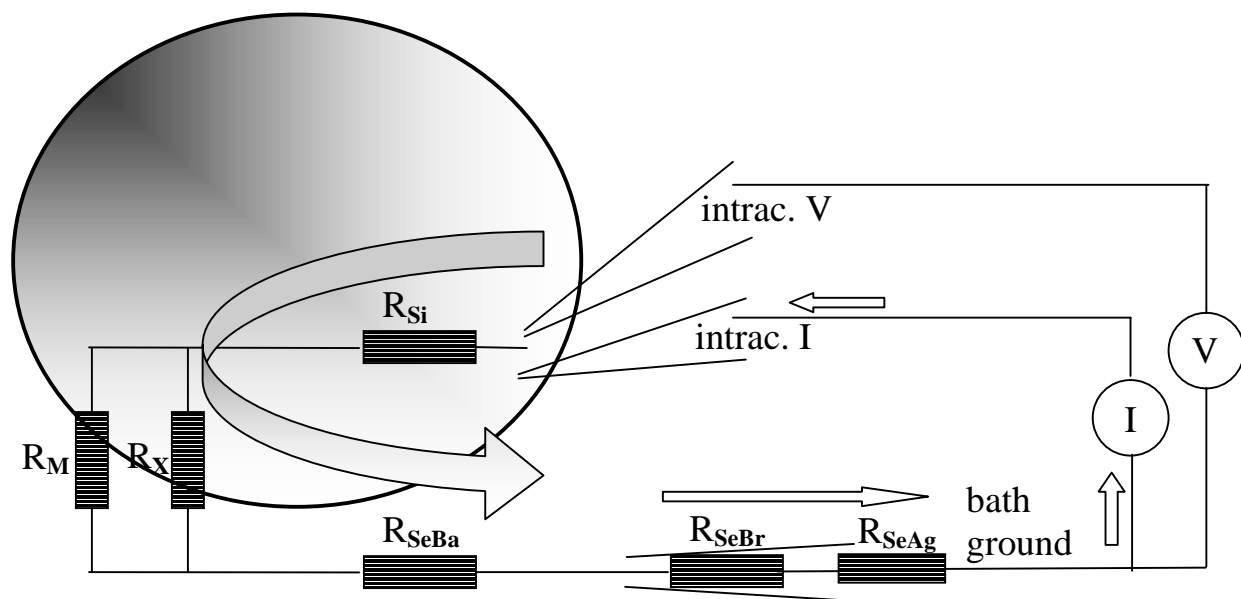
A**B**

Figure 1: Schematic diagram of electrical circuit for Two-Electrode Voltage-Clamp (TEVC). R_M = resistance of oocyte membrane due to endogenous channels and transporters. R_X = resistance of heterologously expressed channels or transporters. R_S = Series Resistance, specifically: R_{Si} = cytoplasmic (internal) series resistance, R_{Se} = extracellular series resistance, composed of R_{SeBa} = resistance of bath fluid, R_{SeBr} = resistance of agar or flowing KCl bridge, and R_{SeAg} = resistance of Ag/AgCl-electrode.

a, electrical circuit with two bath electrodes. **b**, electrical circuit with one bath electrode.

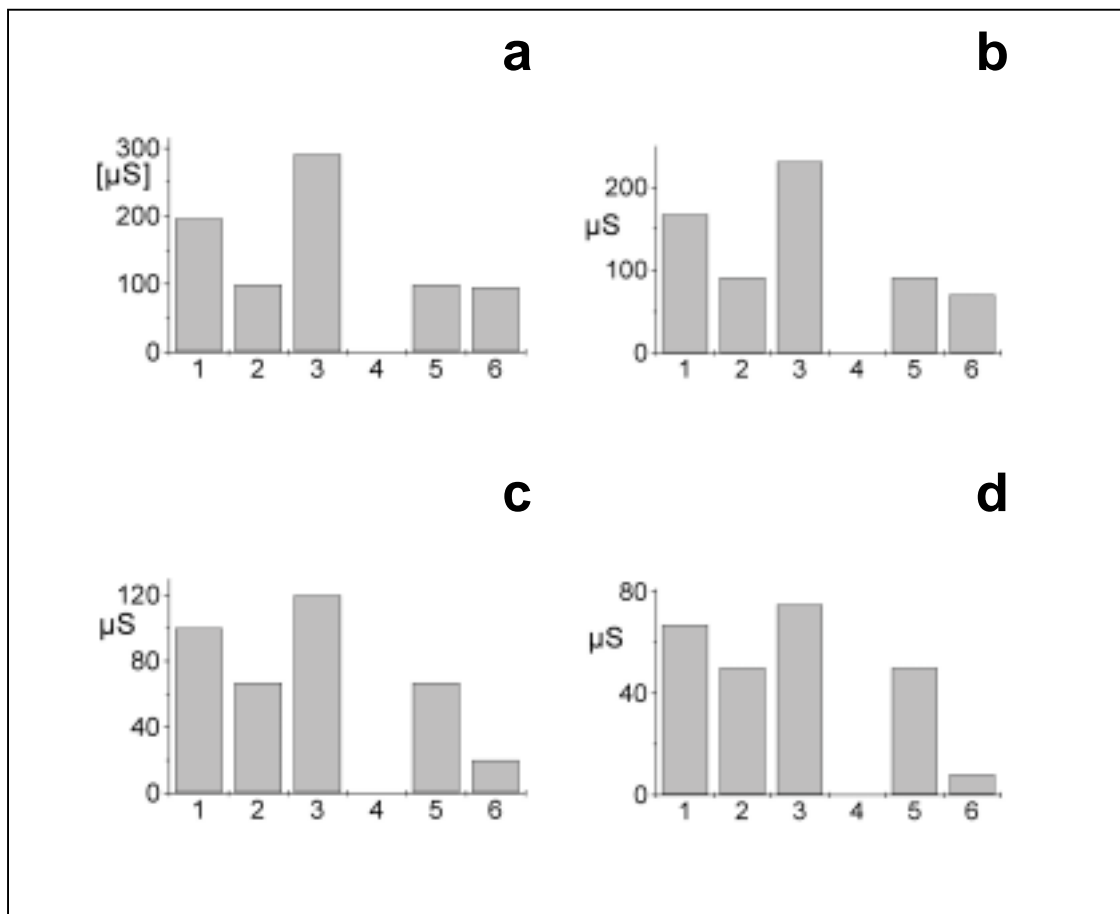


Figure 2 : Apparent CFTR and ENaC conductances, when determined with different series resistances from the same oocyte.

1: Apparent conductance change by activation of CFTR

2: Apparent conductance change by activation of ENaC (i. e. removal of amiloride, before or after CFTR activation)

3: Apparent conductance change by activation of CFTR and ENaC

4: no data, space holder

5: = 2: apparent ENaC conductance when CFTR is inactive

6: = 3 – 1: apparent ENaC conductance when CFTR is activated

a: series resistance of 100 Ω.

b: series resistance of 1 kΩ.

c: series resistance of 5 kΩ.

d: series resistance of 10 kΩ.

Table1: Components of the extracellular series resistance (R_{Se})

Components	Typical resistance	
	One-Bath-Electrode:	Two-Bath-Electrodes:
bath fluid	$R_{SeBa} = 100 \Omega - 10 \text{ k} \Omega$	100 Ω - 10 k Ω
KCl-"bridge" to bath solution	$R_{SeBr} = 0.5 - 10 \text{ k} \Omega$	no contribution
Ag/AgCl-electrode	$R_{SeAg} = \text{several } 100 \Omega$	no contribution
Total $R_{Se} \approx R_S$	1 k Ω - 20 k Ω	100 Ω - 10 k Ω

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