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Improvement of the Accuracy by the Measurement of the Electrical Cell Membrane Parameters

V. ROHLÍČEK, F. RECH

Institute of Physiology, Czech Academy of Sciences, Prague, Czech Republic

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Summary

The electrical parameters of the cell membrane are mostly estimated employing ***ac*** methods. The measurement is based on the analysis of the current(s) flowing through an access resistance and the membrane. A current/potential transducer is used at the input of the device. The parameters of this transducer, especially its feedback capacity, degrades the accuracy of the measurement and hence diminishes the suppression of mutual influences of the individual parameters. The paper suggests a possible software correction and is supplemented by remarks for practical application.

Key words
Patch clamp technique • Electrical parameters of the cell membrane • Membrane capacity • Membrane conductance • Access resistance • Dual frequency method • Exocytosis

Introduction

The first estimation of membrane capacity was described by (Cole 1935), however, his technique did not allow the measurement of small changes of cell membrane capacity. The use of intracellular microelectrodes for electrical measurement was published by Jaffe *et al.* (1978). Just the introduction of the whole cell patch clamp technique together with synchronous detection (Neher and Marty 1982) attained the necessary resolution power for the monitoring of small membrane capacity changes, for instance single events by exocytosis. The detailed analysis of this technique was performed by (Joshi and Fernandez 1988) and the improvements were published by (Lindau and Neher 1988, Fidler and Fernandez 1989, Okada *et al.* 1992). Some drawbacks of these methods (see below) were overcome by introducing a dual frequency method by Rohlíček and Rohlíček (1993) and this method was used for measuring changes of the cell membrane capacity of pancreatic acinar cells (Rohlíček and Schmid 1994). The method was later applied and published by Donnelly (1994), improved by Rech *et al.* (1996) and modified by Barnet and Misler (1997).

The measurement of cell membrane electrical parameters (i.e. membrane capacity (*Cm*) and membrane resistance (*Rm*) or membrane conductivity (*Gm*)) most often employs a patch clamp arrangement in the whole cell configuration. The measurement of electrical membrane parameters is frequently combined with the patch clamp measurement and for both these measurements the same input probe is used. An operational amplifier with a negative feedback in the probe transforms the current flowing through the access resistance and membrane into a proportional potential. It will be shown that at least three parameters of this

Fax+420 2 24920590
E-mail physres@biomed.cas.cz
http://www.biomed.cas.cz/physiolres

configuration (feedback stray capacity, open loop gain and transient frequency of the amplifier) negatively influence the proportionality between the input current and the output potential as far as the amplitude and phase are concerned. This causes a degradation of the accuracy of the evaluation as well as of the suppression of the mutual influence between the individual measured parameters which can introduce artifacts.

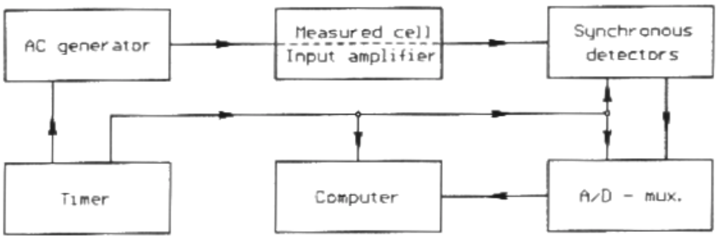

Fig. 1. *Arrangement of the measurement.*

Methods

Figure 1 presents a typical arrangement for the measurement. The timer uses a crystal controlled oscillator for generation of the following signals: control signals for the AC generator, switching potentials for synchronous detectors, control signals for the computer and an A/D transducer and multiplexer. The signal from the AC generator is applied to the cell and the current/potential conversion is performed in the input amplifier (block „Measured cell - Input probe in Fig. 1). A detailed scheme of this part is presented in Fig. 2. The synchronous detectors offer potentials proportional to the real and imaginary components of the output of the probe. At the input of the measuring apparature (probe) an *i-u* (current - potential) transducer is used. In most cases a probe of a patch clamp device serves as this transducer.

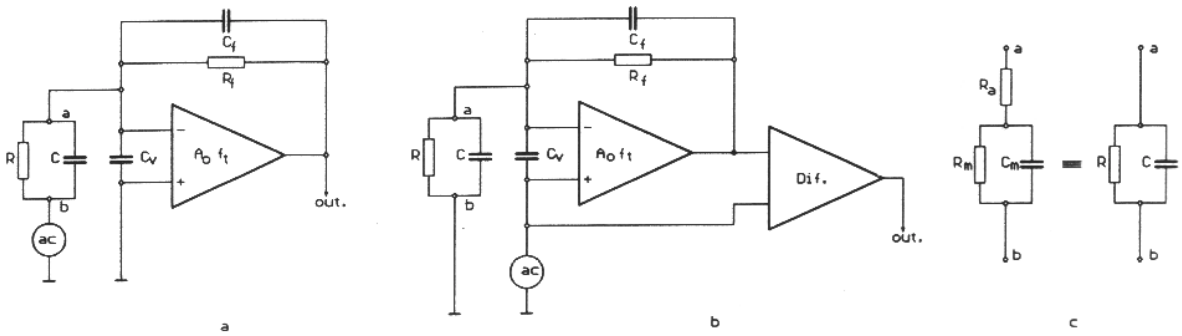

Fig. 2. *Possible input combinations, a) inverting combination, b) non-inverting combination, c) transformation of input admittance. R,C = transformed input admittance, ac = ac generator, Cv = input capacity of the i/e converter, Ao, ft = internal constants of the i/e converter, Cf, Rf = feedback parameters of the i/e converter, dif = differential amplifier.*

Figs. 2a and 2b offer two possibilities of the connection of the input probe. The solution 2a is more simple (a differential amplifier is not necessary). Its disadvantage is that an ***ac*** potential in the bath requires an ***ac*** source with minimal internal resistance. The advantage of the connection 2b is the grounded bath. In most connections of this type an input dual FET transistor serves as a differential amplifier with emitters connected to a current source (Sigworth 1983). We assume that the input capacity is compensated in both cases.

An admittance formed by any combination of resistors and capacities connected to the input terminals *a* and *b* (Fig. 2c) can be expressed with real (*Re*) and imaginary (*Im)* components. For the special case of a serial connection of a resistor (*Ra*) with a parallel combination of the resistor *Rm* and a capacity *Cm* which is typical for measurements of electrical cell membrane parameters, both components are given by the following equations:

$$Re = 1/Rm.\frac{1 + Ra/Rm + \omega^2.Cm^2.Ra.Rm}{(1 + Ra/Rm)^2 + (\omega.Cm.Ra)^2} \qquad (1)$$

$$Im = \frac{\omega.Cm}{(1 + Ra/Rm)^2 + (\omega.Cm.Ra)^2} \qquad (2)$$

where: Ra = access resistance, Rm = membrane resistance and Cm = membrane capacity Re is the real part, $1/Re$ then the tranformed resistance R and Im the imaginary part of the admittance between terminals *a* and *b,* the transformed capacity C equals Im/ω. Note that the transformation is valid only for one particular frequency.

A potential proportional to this admittance occurs at the output of the *i/u* transducer only under the assumption that the input capacity is compensated, there is no stray capacity in the feedback loop ($Cf=0$), the amplification and transient frequency of the amplifier are so high, that their influence on the *i/u* conversion can be neglected and that the input capacity is compensated.

Under real conditions, when a commercial patch-clamp device is used for the input capacity compensation, any other compensations have to be switched off. The real component Re and imaginary component Im of the admittance both change at the output of the *i/u* transducer in I (inphase component) and Im in Q (quadrature component). A *dc* potential proportional to I and/or Q then occurs at the output of synchronous detectors the switching signal of which has a phase relation to the excitation potential strictly 0 or $\pi/2$.

$$I = \frac{\alpha . A + \beta . B . \omega^2}{A^2 + \omega^2 . B^2} \tag{3}$$

$$Q = \omega \frac{A . \beta - B . \alpha}{A^2 + \omega^2 . B^2} \tag{4}$$

where :

$$\alpha = Re . Rf - \frac{1 + Rf . Re}{Ao} + \frac{\omega^2 . Rf . (Cf + Cv + Im/\omega)}{\omega t} \tag{5}$$

$$\beta = Rf . Im/\omega - \frac{Rf}{Ao} . (Cf + Cv + Im/\omega) - \frac{1 + Rf . Re}{\omega t} \tag{6}$$

$$A = 1 + \frac{1 + Rf . Re}{Ao} - \frac{\omega^2 . Rf . (Cf + Cv + Im/\omega)}{\omega t} \tag{7}$$

$$B = Rf . Cf + \frac{Rf . (Cf + Cv + Im/\omega)}{Ao} + \frac{1 + Rf . Re}{\omega t} \tag{8}$$

where: Rf = feedback resistance; Cf = feedback capacity; Cv=input capacity of the amplifier; ωt = transient frequency of the amplifier; Ao= open loop gain of the amplifier.

These expressions are valid for a noninverting connection. For an inverting connection only α and β changes into :

$$\alpha = Rf . Re \tag{5a}$$

$$\beta = Rf . Im/\omega \tag{6a}$$

The presence of the frequency ω in the equations signifies that the correction is valid only for one given frequency. This must be taken into account by the application of dual or multifrequency methods.

Re and Im defined by (1) and (2) are simultaneous variables in a system of two non-linear equations:

$$F(Re, Im) \equiv I - Io = 0 \tag{9}$$

$$G(Re, Im) \equiv Q - Qo = 0 \tag{10}$$

where Io and Qo are the measured values (outputs from the synchronous detectors). I and Q are defined by equations (3) and (4) and represent a mathematical model of real and imaginary parts of the transfer under real conditions.

Solution for Re and Im of non linear equations (9) and (10) can be performed only by an approximation to:

$$Re_k = Re \tag{11}$$

$$Im_k = Im \tag{12}$$

where Re_k is the real component of the measured admittance after correction, Im_k is the imaginary component of the measured admittance after correction.

The solution has to fulfil the following condition:

$$|F| + |G| \leq \varepsilon \tag{13}$$

where ε is small (we have used $\varepsilon = 10^{-10}$)

Such a system of non-linear equations can be solved by several methods (Ralston 1973, Nekvinda 1976, Vitásek 1987).

We have selected the Newton method, because we believe that this method has the smallest number of iteration steps for our purpose due its quadratic convergence and thus saves computer time. The computer model has shown, that in most cases the number of iteration steps is 3, rarely 2 or 4.

For the first step of the solution it is necessary to estimate the approximate values of Re and Im .

Instead of beginning with fixed values of *Re* and *Im* in the centre of the range, we found that the nearest approximation is to use the values given by the output of the synchronous detectors *I* and *Q*.

The reconstruction of the original parameters *Ra, Rm* and *Cm* depends on the method employed. The methods which use one sinusoidal frequency under-estimate the system because only two equations are at our disposal for the three variables. Therefore, a third relation has to be found. For instance, a *dc* potential was superposed on the sinusoidal potential (Lindau and Neher 1988). This technique assumed a constant and known reversal potential of the cell during the whole experiment. Possible electrical stimulation of the cell by a *dc* component of the excitation potential cannot be excluded. A phase tracking technique published by (Fidler and Fernandes 1989) assumes that the cell parameters are approximately constant during the experiment. Another approach was the superposition of a slow (2 Hz) rectangular potential to the sinusoidal measuring potential (Okada *et al.* 1992). This technique does not allow registration of abrupt changes of the membrane parameters. The mathematical derivation of the original parameters is described in papers mentioned above.

To overcome the drawbacks of monofrequency methods a dual frequency method was developed (Rohlíček and Rohlíček 1993). We applied the correction described for this method. In this case, it was necessary to correct the output of the synchronous detectors for both frequencies.

To obtain the original parameters the following equations were used :

$$Cm = c.\frac{k.a_2.\left(a_3^2 + a_4^2\right) - a_4.\left(a_1^2 + a_2^2\right)}{\left(k^2 - 1\right).\omega.a_2.a_4} \tag{14}$$

$$Rm = \frac{\frac{1}{c}.\left(k^2 - 1\right).a_2.a_4}{k.a_1\left(a_3^2 + a_4^2\right) - a_3.\left(a_1^2 + a_2^2\right)} \tag{15}$$

$$Ra = \frac{\frac{1}{c}.\left(k.a_2.a_3 - a_1.a_4\right)}{k.a_2.\left(a_3^2 + a_4^2\right) - a_4.\left(a_1^2 + a_2^2\right)} \tag{16}$$

where a_1 - a_4 are the output potentials of the synchronous detectors :(Re_k and Im_k); a_1 is a real and a_2 an imaginary component for the low frequency, a_3 is a real; a_4 an imaginary component for the high frequency; ω is the low frequency and k is the ratio of both frequencies; c is a coefficient of proportionality respecting the value of the measuring potential and the amplification in the evaluating chain.

Results

The tests were performed on a) a computer model and b) a model circuit. Both were used for testing all combinations of values *Rs* = 1, 2, 4, 8 MΩ, *Rm* = 100, 200, 400, 800, 1600 MΩ, *Cm* = 5, 10, 15 pF (which, as we believe, cover the relevant range).

a) The computer model allowed us not only to verify the mathematical procedure, but it also made it possible to investigate rapidly and accurately the influence of an incorrect estimation of any component in the feedback loop, as well as of the parameters of the amplifier.

b) As a model circuit, the connections shown in Fig. 2a and 2b were constructed with scaling of the resistances 1/1000 and capacities 1000/1. This arrangement of the model was used for the following reasons:

1. It would be nearly impossible for the original values, in the range of interest mentioned above, to construct a switching arrangement by which changes of the capacity during switching would be smaller than 5 fF (including differences in the stray capacity on individual components).

2. An accurate calibration of the component would only be possible by using special measuring devices .

3. The noise by the scaling described above is much smaller and therefore the measurements are more accurate.

The comparison with values measured with the model circuit have shown good agreement with the measurements on cells.

The application of the method described requires knowledge of the values of the feedback components and parameters of the amplifier. In the technical data summarized in the handbook delivered along with the device, only values of the feedback resistance *Rf* are reported. Other values, i.e. feedback capacity, open loop gain and transient frequency of the amplifier have to be estimated. The most frequent artifact when measuring the cell membrane capacity is the influence of *Cm* indicated by changes in cell membrane resistance which can easily exceed 100 % (Rohlicek and Schmid 1994). Therefore, we paid attention to the problem how the incorrect estimation of the feedback capacity *Cf*, open loop gain of the amplifier A_0 and the transient frequency of the amplifier *ft* influence the suppression of this artifact.

For this purpose we used a standard sensitivity function, for example that proposed by Di Stefano et *al.* (1967).

$$C = \frac{Rm1}{Cm1} * \left\| \frac{Cm2 - Cm1}{Rm2 - Rm1} \right\| \tag{17}$$

We are using the reciprocal value of the sensitivity function, i.e. the ratio of the relative change of resistance *Rm* to the relative change of capacity *Cm* which expresses the suppression *Sf* of the artifact.

The analysis was performed for the following parameters: the low frequency (500 Hz) and high frequency (1 kHz), the open loop gain of the amplifier $A_0 = 10^5$, its transient frequency ft = 10 MHz, the feedback resistance Rf = 500 MΩ and the feedback stray capacity Cf = 100 fF.

Tables 1 to 3 show how critical is the estimation of the values Cf, A_0 and *ft.*

Table 1.

Ra MΩ	Rm MΩ	Cm pF	*Sf*	Δ Cf
1	1600	5	2500	-3 %
8	50	15	227	-3 %
1	1600	5	5000	-1 %
8	50	15	458	-1 %
1	1600	5	5000	+3 %
8	50	15	517	+3 %
1	1600	5	2500	+1 %
8	50	15	263	+1 %

Table 2.

Ra MΩ	*Rm* MΩ	*Cm* pF	*Sf*	*Δ ft*
1	50	15	1500	-20 %
8	1600	5	$>10^4$	-20 %
1	1600	5	$>10^4$	+50 %
8	50	15	7500	+50 %

First were calculated in phase and quadrature values in the whole relevant range, i.e. *Rs* = 1, 2, 4, 8 MΩ, *Rm* = 100, 200, 400, 800, 1600 MΩ, *Cm* = 5, 10, 15 pF.

In the second step correction was performed with incorrect setting of *Cf*, A_0 and *ft* as indicated in the last columns of the tables. All combination of values in the range of interest were taken into account and the worst case conditions were estimated and presented in rows. The odd rows are for positive disagreement from the correct value, the even rows for the negative one.

The last column shows the difference from the proper values of the respective parameters

The factor *Sf* illustrates the suppression of the change of membrane resistance to the membrane capacity and is a reciprocal value of the sensitivity function.

The curves in Fig. 3. illustrates the change of the suppression factor as a function of the value of membrane resistance *Rm* in the middle range, i.e. for capacity Cm = 10 pF and access resistance Ra= 2 MΩ and disagreement of the capacity *Cf* = +/- 1 and 3 %.

Table 3.

Ra MΩ	*Rm* MΩ	*Cm* pF	Sf	*Δ Ao*
1	1600	5	$>10^4$	-50 %
8	50	15	$>10^4$	-50 %
1	1600	5	$>10^4$	+100 %
8	50	15	$>10^4$	+100 %

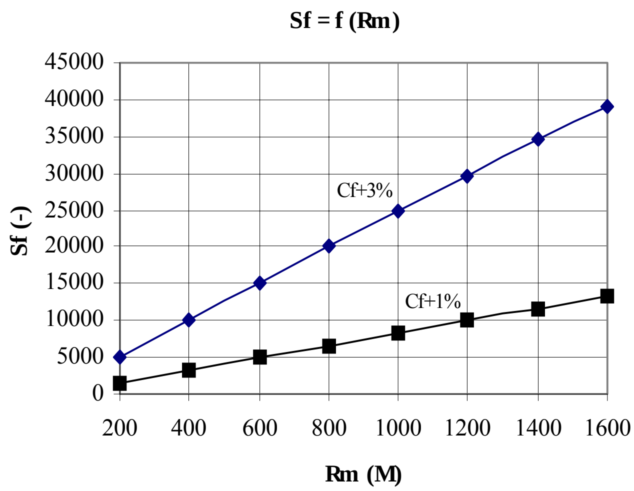

Fig. 3. *Decrease of the suppression factor Sf by incorrect setting of the feedback capacity Cf.*

Discussion

Regardless of the method used for measurement of electrical membrane parameters, the measurement starts with calibration of the apparatus. This calibration is mostly performed by connecting the probe with a model circuit (dummy) the electrical parameters of which are known. This calibration includes the compensation of all influences described above. A discussion of the equations (1-4) shows that the distortion introduced by the mentioned parameters of the apparatus depends on the actual values of *Ra, Rm* and *Cm*. In other words, the calibration compensates all influences only for just one particular case. The admittance of the combination of Ra, *Rm* and *Cm* represent a vector. By a dual or higher frequency method it is necessary to consider admittances as vectors. If another combination occurs the vector(s) are different. The greater the difference, both in the angle as well as in the amplitude of this (these) vector(s), the lower the compensation of the influences mentioned above. This leads to the following results:

a) Inaccuracy of the absolute values of the parameters measured. In most of the real conditions this inaccuracy does not exceed several percent, which is not very important because of the differences of these parameters in different cells of the same type if these cells also belong to the same sample.

b) Of considerable importance is the degradation of the suppression of the influence exerted by the change in membrane resistance on the indicated capacity (suppression factor) in case of another combination compared to that for which the calibration was performed. Such abrupt changes in membrane resistance can exceed 100 % as shown by Rohlíček and Schmid (1994). Taking into account that the changes in membrane capacity of interest (single events of exocytosis, endocytosis, granulation, fertilization etc.) are in the range 10^{-3} of *Cm* (each single event by about 10 fF of the total membrane capacity of 10 pF), the suppression factor has to be at least 1000, otherwise there arises danger of artifacts which cannot be neglected.

The above method has the following advantages:
a) Only one calibration is necessary which is valid for all input admittances without loss of the suppression factor thus minimizing the artifacts.
b) No model circuits are necessary for the calibration. It is sufficient to connect the inputs of the synchronous detectors with the output of the generator of measuring potentials and to set the in-phase potential to 1 and quadrature one to zero.

As mentioned above, it is necessary for the application of the method to insert the values of *Rf, Cf, Ao, ft* into the equations. In the technical data of the patch-clamp apparatus only *Rf* values are usually given.

When looking at Tables 1, 2 and 3 it is possible to conclude that the open loop amplification *Ao has* the smallest influence on the correction process and that in most cases we may assume this value to be 5.10^4 without introducing a significant error. The estimation of the transient frequency is more important and at least a rough assessment is necessary. The accurate estimation of the feedback capacity is of paramount importance. Table 1 shows that an accuracy better then 3 % is desirable. The same accuracy has to apply to the value of *Rf.* In this case, a verification of the value published in the manual is to be recommended. In the Appendix, some of the methods for measurement of the mentioned parameters are described.

During practical application of the system (Rohlíček and Schmid 1994) a negative influence of distributed parameters of the pipette was not observed.

The mathematical derivation of the most equations in this paper would be out of the scope of this journal. A theoretical paper with the complete mathematical derivation is under preparation. The readers interested in this part of the present communication should contact the senior author of this presentation (V. R.).

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Appendix

Measurement of the feedback capacity *Cf* and the parallel input capacity *Cp*

The most important parameter for the correction software is the value of the feedback capacity *Cf.* A direct measurement is almost impossible, because the greatest part of this capacity forms the stray capacity of the feedback resistor Rf which is in most cases an intrinsic part of the customer-integrated circuit in the probe. We developed a method for the indirect measurement of this capacity which also allows the estimation of a parallel input capacity (*Cp).*

This method only uses one frequency, so that it can be used for all *ac* methods for measuring the electrical parameters of the cell membrane. For these measurements, an auxiliary circuit consisting of a parallel combination of a capacity (C_1) and a resistor (R_1) is connected to the input of the probe (points *a* and *b* in Fig. 2a and 2b.). The estimation of *Cf* and *Cp* is based on the solution of the following equations (Ralston 1965, Nekvinda 1976).

$$F(x,y) \equiv I - Io = 0 \tag{18}$$

$$G(x,y) \equiv Q - Qo = 0 \tag{19}$$

where

I_0 *and* Q_0 are measured inphase and quadrature components,

x and *y* are auxiliary variables given by

$$x = Rf.(C_1 + Cp), \Rightarrow Cp = x/Rf - C_1 \tag{20}$$

$$y = Rf.Cf \Rightarrow Cf = y/Rf \tag{21}$$

$$I = \frac{\alpha.A + \beta.B.\omega^2}{A^2 + \omega^2.B^2} \tag{22}$$

$$Q = \omega\frac{A.\beta - B.\alpha}{A^2 + \omega^2.B^2} \tag{23}$$

where:

$$A = A_0 - \frac{\omega^2}{\omega_t}.(x+y) \quad A_0 = 1 + \frac{1+\frac{Rf}{R_1}}{k_0} - \frac{\omega^2}{\omega_t}.Rf.Cv \tag{24}$$

$$B = B_0 + \frac{x}{k_0} + y.\left(1+\frac{1}{k_0}\right) \quad B_0 = \frac{1+\frac{Rf}{R_1}}{\omega_t} + \frac{Rf.Cv}{k_0} \tag{25}$$

$$\alpha = \alpha_0 + \frac{\omega^2}{\omega_t}.(x+y) \quad \alpha_0 = 1 + \frac{Rf}{R_1} - A_0 \tag{26}$$

$$\beta = \beta_0 + x.(1 - \frac{1}{k_0}) - \frac{y}{k_0} \quad \beta_0 = -B_0 \tag{27}$$

The solution of equations 18 and 19 is performed by the Newton method (Vitásek 1987). The following expression is used as the first approximation:

$$x_0 = (I_0^{\,2} + Q_0^{\,2})/(\omega\,.\,ABSQ_0)\ ;\ \ y_0 = I_0/(\omega\,.\,ABSQ_0) \tag{28}$$

Because this measurement is only performed once for a given apparatus, it is possible to compare the values after averaging 100 times.

Measurement of the feedback resistor R_f

The value of the feedback resistor R_f is of similar importance as the value of the feedback capacity C_f because for the accurate correction they represent together the feedback impedance (or feedback time constant $\tau_f = C_f\,.\,R_f$).

The value of this resistor is mostly given in the data sheet of the patch-clamp device, however, usually without the required accuracy of this value.

Fortunately, the measurement of the feedback resistor *Rf* is relatively easy. It is sufficient to connect an auxiliary resistor R_{aux} of a known (accurate) value to the input terminal of the probe and the other terminal of the resistor to a known *dc* potential and measure the output potential of the *i /e* transducer R_f is then given by:

$$R_f = R_{aux}\,.\,u_{in} / u_{out}$$

where

R_{aux} is the auxiliary resistor

u_{in} the known *dc* potential

u_{out} the output potential of the probe.

Measurement of the transient frequency f_t.

The direct measurement of the utilize a frequency f_t is, not possible owing to the unavoidable capacity C_f. Therefore, we can use the property of an operational amplifier the amplifying factor of which decreases with the frequency from the open loop gain A_0 to the factor 1 with a slope of -20 dB/dek (Dostál 1981).

For a negative feedback, the amplification is given by:

$A = R_f / R_1$ inverting input,

$A = 1 + R_f / R_1$ non - inverting input.

R_f is the feedback resistor between the input and output terminals of the amplifier and R_1 is a resistor connected between the terminal of the amplifier and zero potential.

It is sufficient to estimate the frequency for the 3 dB point (corner frequency f_c). The transient frequency is as follow:

$$f_t = f_c \cdot A$$

A small value of the resistor R_f should be selected compared to the feedback capacity C_f.

Table 2 shows that this measurement is not very critical, an error of 20 % is acceptable.

Measurement of the open loop gain A_0

Most methods for this measurement utilize a resistance divider R_3 / R_4 (Fig. 4.). If $R_1 = R_2$ the open loop gain is :

$$A_0 = (R_3/R_4 + 1).e_{out} / e_d$$

In most marketed devices it is not possible to insert the divider R_3 / R_4. In this case (R_3 = 0; R_4 omitted) the potential e_d *is* equal to the potential between the input terminals of the amplifier e_d. If $R_1 = R_2$ the open loop gain is given by:

$$A_0 = e_{out}/e_d$$

To exclude the influence of the feedback capacity (not drawn), the measurement should be performed either with a *dc* potential or with an *ac* potential the frequency of which is below the first corner frequency of the amplifier.

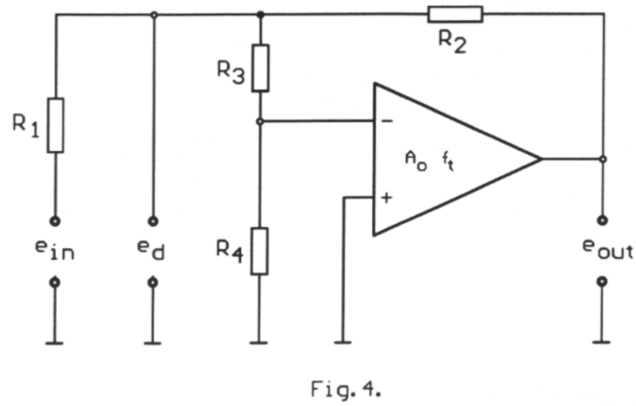

Fig. 4. *Arrangement for measurement of the Ao of the i/e transducer.*

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Reprint requests

V. Rohlíček, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic. E-mail : Rohlicek@biomed.cas.cz