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Georges Christé

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LOCALIZATION OF  $K^+$  CHANNELS IN THE T-TUBULES OF  
CARDIOMYOCYTES AS SUGGESTED BY THE PARALLEL  
DECAY OF MEMBRANE CAPACITANCE,  $IK_1$  AND  $IK_{ATP}$   
DURING CULTURE AND BY DELAYED  $IK_1$  RESPONSE TO  
BARIUM.

Georges Christé

Unité de Recherche sur l'Activité Electrique du Coeur, INSERM U 121, 22 Ave  
Doyen Lépine, 69500 BRON, France

Correspondence and reprints to actual address:

Georges CHRISTÉ  
Groupe d'Electrophysiologie Moléculaire,  
Laboratoire de Bioénergétique Fondamentale et Appliquée,  
Université Joseph Fourier,  
B.P. 53 X,  
F-38041 Grenoble CEDEX 9  
Tel: (33) 4 76 51 43 05 Fax: (33) 4 76 51 42 18  
Email: Georges.Christe@ujf-grenoble.fr

Short title: **Inward rectifier currents and cardiac T-tubules**

**Abstract**

Adult ventricular myocytes lose T-tubules over few days in culture, which causes the loss of about 60% of the cell membrane capacitance ( $C_m$ ) (Mitcheson *et al.* 1996). In this study, we have measured, in whole-cell voltage-clamped rabbit  
5 right ventricular myocytes at 0, 1, 2 and 3-5 days of culture (nine to 20 myocytes at each age) in a defined (DMEM) medium, the value of  $C_m$  and the magnitudes of the background inward rectifier current ( $IK_1$ ) and of the 2,4-dinitrophenol-induced ATP-sensitive potassium current ( $IK_{ATP}$ ).  $C_m$ ,  $IK_1$  and  $IK_{ATP}$  all had  
10 decreased significantly by 51, 83 and 88%, respectively after 4 days of culture. Analysis using a single exponential decay function of time gave time constants of respectively  $2.6 \pm 0.2$ ,  $2.2 \pm 0.5$  and  $2.4 \pm 0.4$  days, respectively. Linear regressions of  $IK_1$  and  $IK_{ATP}$  versus  $C_m$  had regression coefficients of 0.93 and 0.98, respectively. These observations are consistent with a strong link of the decay of  $IK_1$  and  $IK_{ATP}$  currents to that of  $C_m$ . Furthermore, the time course of changes in

IK<sub>1</sub> when an external blocker (100 μM BaCl<sub>2</sub>) was applied and washed by local perfusion (95% change in 50 ms) agrees with a model including a diffusion time constant of 300 ms. This value is consistent with the known kinetics of diffusion of divalent cations in the T-tubules.

- 5 Taken together, these results could be explained by the localization of a major part of the IK<sub>1</sub> and IK<sub>ATP</sub> currents of ventricular cardiomyocytes in the T-tubules. As a consequence, transient accumulation of K<sup>+</sup> ions in cardiac T-tubules may take place and modulate excitation-contraction coupling.

10

**Key words:** T-tubule; Background inward rectifier K channels; ATP-sensitive K channels; Membrane compartmentation.

## Introduction

Potassium currents are important determinants of the electrical activity of cardiac ventricular muscle (for a review: Carmeliet, 1993). The background inward  
5 rectifier current ( $IK_1$ ) governs the resting potential and contributes to the late phase of action potential repolarization, while the ATP-sensitive potassium current ( $IK_{ATP}$ ) current is involved during ischemic/hypoxic challenge to the myocardium. These currents also contribute to accumulation-depletion of potassium in extracellular spaces, either in the physiological state or in  
10 pathological states. In skeletal muscle, the localization of  $K^+$  conductances in the T-tubule membrane leads to accumulation-depletion of  $K^+$  ions that have important consequences on muscle function (Almers and Stirling, 1984, for review). In cardiac muscle, such consequences may not yet be evaluated, as the membrane distribution of  $K^+$  conductances is not known. It was recently  
15 established that ventricular cardiomyocytes in primary culture lose their T-tubules and the associated membrane capacitance (Lipp *et al.*, 1996; Mitcheson *et al.*, 1996). We report here that cultured rabbit ventricular myocytes lose a large part of  $IK_1$  and  $IK_{ATP}$  currents in parallel with the reduction in cell membrane capacitance ( $C_m$ ). Moreover,  $IK_1$  changes upon rapid external application and  
20 washout of  $Ba^{++}$  ions occur with a initial delay consistent with a model of diffusion-limited access to the  $IK_1$  channels. These data provide arguments for the preferential localization of  $IK_1$  and  $IK_{ATP}$  conductances to the T-tubule membrane. The functional consequences of this localization are discussed.

25

## Materials and Methods

Materials and methods, unless otherwise specified, are the same as previously described (Christé *et al.* 1999).

30

### *Isolation of Cardiomyocytes*

Under sterile conditions the heart of New-Zealand female rabbits weighting 1.5 to 1.7 kg were excised under deep anaesthesia (Xylazine 20 mg.kg<sup>-1</sup> plus  
35 Ketamine 100 mg.kg<sup>-1</sup>) and placed at the bottom of a Langendorff perfusion system. Ventricular myocytes were isolated by a conventional enzymatic digestion method and cultured in DMEM medium. The medium was renewed every other day.

The investigation conforms with the Recommendation from the Declaration of  
40 Helsinki and the Guiding Principles in the Care and Use of Laboratory Animals.

The "external solution" for whole-cell current recording experiments contained (mM): NaCl 150; KCl 5.4; MgCl<sub>2</sub> 1.2; CaCl<sub>2</sub> 0.2; NaH<sub>2</sub>PO<sub>4</sub> 0.905; HEPES 10; glucose 10; pH was adjusted to 7.4 with NaOH. The pipette solution contained (mM): K-aspartate 115; KCl 5; MgCl<sub>2</sub> 7; K<sub>2</sub>-ATP 4; Na<sub>2</sub>-phosphocreatine 2; pyruvic acid 5; Na<sub>2</sub>-EGTA 5; HEPES 10, pH was adjusted to 7.2 with KOH. DMEM medium was prepared from powder (GIBCO-Life Sciences Technologies) and supplemented with penicillin-G 60 mg/l, streptomycin 50 mg/l and NaHCO<sub>3</sub> 25 mM. No serum or growth factor additives were used. All chemicals, including 2,4-Dinitrophenol (DNP) were of analytical grade and purchased from Sigma (Saint Louis, MO).

### *Whole cell current measurements*

Cells were placed on a glass coverslip that constituted the bottom of a narrow well (3 x 15 mm) machined in a water-jacketed perspex block. The inflow of physiological solution was pre-warmed through a 10 cm water jacket. Its temperature was maintained at 35 ± 1 °C (as assessed using a 0.1 mm thermocouple) by circulating the water jackets with thermostated water. Whole cell currents were monitored with a List EPC5 patch-clamp amplifier. Experiments were driven and recorded by a TL1 DA/AD interface and PClamp program suite (Axon Instruments). Cell capacitance (C<sub>m</sub>) and series resistance (R<sub>s</sub>) were evaluated as follows. Twenty current responses to ΔV=10 mV square depolarizing pulses of 12 ms duration were sampled at 25 KHz and averaged. A single exponential was fitted to the capacitance current decay, which provided estimates of the initial current **I<sub>0</sub>**, the time constant **Tau** and the steady state current **I<sub>ss</sub>**. C<sub>m</sub> was derived using the relation:

$$C_m = \text{Tau} * (1/R_s + 1/R_m)$$

with

$$R_s = \Delta V / I_0 \quad \text{and} \quad R_m = (\Delta V / I_{ss} - \Delta V / I_0).$$

A voltage-clamp ramp protocol was used for recording membrane currents. From a holding potential of -80 mV, a square prepulse to -120 mV for 25 ms was followed by a depolarizing voltage ramp from -120 to +120 mV (2.5 mV.ms<sup>-1</sup>) and a reverse ramp from +120 to -120 mV. The whole cell current data in response to the repolarizing ramp were corrected off-line for estimated junction potentials, R<sub>s</sub>-induced drop from command voltage and ramp-induced capacitance current as previously described (Christé et al. 1999).

The main artefact was due to real R<sub>s</sub> values ranging 5 to 15 MΩ, which caused large departures of real voltage from the command voltage ramp. After corrections were applied to both the recordings in the absence of DNP (control) and in its presence, the magnitude of the maximal DNP-induced current representing I<sub>K<sub>ATP</sub></sub> was measured at 0 mV after subtraction of control current.

To isolate the  $IK_1$  current, a linear leakage current reversing at 0 mV was computed and subtracted, yielding the corrected current versus voltage relations as averaged and plotted in figure 1A. The leakage conductance was estimated for each cell using the current magnitude at the observed reversal voltage of the current suppressed by 100  $\mu$ M  $BaCl_2$  (i.e. -73 to -75 mV). The magnitude of the  $IK_1$  current for each cell was then measured as the absolute difference in currents at -60 and -90 mV.

#### *Data analysis and statistics*

Membrane current data were processed under Clampfit and custom programs in Fortran-77 and MATLAB (The Math Works Inc.). Statistical analysis and graphs were done under ORIGIN 4.1 (Microcal Inc.). Data are given as mean  $\pm$  S.E.. Comparison of means used unpaired t-test. The level of statistical significance was the 95% confidence level.

The time-dependent decay of cell capacitance or whole cell current magnitudes during culture was evaluated with an exponential function  $Y(t)$  of the form

$$Y(t) = (Y_0 - Y_{inf}) \cdot \exp(-t/\tau) + Y_{inf} \quad (\text{equation 1})$$

where  $Y_0$  is the value of  $Y(t)$  at time 0,  $\tau$  its time constant of decay and  $Y_{inf}$  its steady-state value. This function was adjusted to the data using a least squares fitting routine. A linear regression routine was built from the Linfit algorithm of Bevington (1969) under MATLAB.

Fitting of equation 1 to data, as well as linear regression, took into account errors both in abscissa and ordinate data sets. Thus, the 95% confidence intervals that were computed for each estimated parameter value include the contribution of both errors from all data points. They are indicated near to parameter values in the text.

#### *Theoretical model of $Ba^{++}$ diffusion and binding to the $IK_1$ channel*

Changes in  $Ba^{++}$  concentration in the T-tubules ( $[Ba]_t$ ) upon changing the bath concentration ( $[Ba]_e$ ) were represented by differential equation:

$$d[Ba]_t/dt = ([Ba]_e - [Ba]_t) / \tau_T \quad (\text{equation 2})$$

where  $\tau_T$  was the time constant of exchange between bulk solution and T-tubule lumen. The fraction of available  $IK_1$  channel ( $F_t$ ) with binding of  $Ba^{++}$  ions to a single external site was computed as:

$$dF_t/dt = k_2 - (k_1 \cdot [Ba]_t + k_2) \cdot F_t \quad (\text{equation 3})$$

where  $k_1$  and  $k_2$  are microscopic binding and unbinding rate constants.

Computation of the theoretical time course of unblocked  $IK_1$  fraction was performed by 4th order Runge Kutta integration under MATLAB.

## Results and Discussion

### *Features of $IK_1$ and $IK_{ATP}$ currents in freshly dissociated and in cultured cells*

Figure 1A shows the average whole cell magnitude of the  $IK_1$  current versus  
 5 voltage in freshly dissociated myocytes (curve a). The current-voltage relation  
 was similar to that reported by Giles and Imaizumi (1988). The  $IK_1$  current was  
 dramatically decreased after 3-5 days in culture conditions (curve b) and in a  
 uniform way over the voltage range considered. A small outward current present  
 at voltages positive to -20 mV was also decreased (Mitcheson et al. 1996).  
 10 The current induced by 100  $\mu$ M DNP (Fig 1B) was fully blocked by 10  $\mu$ M  
 Glibenclamide, was weakly inward rectifying, reversed around -75 mV, thus it  
 was termed  $IK_{ATP}$ . Its magnitude was greatly decreased after more than 3 days in  
 culture (fig 1B). For both currents, the shape of the current-voltage relations were  
 poorly affected by time in culture.

15

### *Decay of membrane capacitance, $IK_1$ and $IK_{ATP}$ currents with time in culture*

As shown in figure 2A, the whole cell capacitance progressively decreased with  
 20 time in culture. From an initial value of  $109 \pm 18$  pF (n=10) on day 0, it decayed to  
 $53 \pm 8$  pF (n=9) after about 4 days. The decay of  $C_m$  was not significant at day 1,  
 and became significant at later times. The loss of cell capacitance fitted with an  
 exponential function (equation 1, solid line in Fig. 2A) proceeded with a time  
 constant  $\tau = 2.6 \pm 0.2$  days (parameter estimate  $\pm$  95% confidence interval), and  
 25 the estimated steady-state level ( $41.4 \pm 2.3$  pF) was not reached after 5 days in  
 culture. Such a decay of membrane capacitance in cultured ventricular myocytes  
 has been mainly correlated with the disappearance of the T-tubules (Lipp *et al.*,  
 1996; Mitcheson *et al.*, 1996) while a minor part was due to decrease in cell size  
 (Mitcheson *et al.* 1996). The fraction of  $C_m$  lost as computed from the present  
 30 data (0.62) agrees with the fraction of the cell membrane in the T-tubules as  
 determined in ventricular myocytes by morphometric methods: 0.53 (Amsellem *et*  
*al.*, 1995).

In about 4 days of culture,  $IK_1$  (Fig. 2B) and  $IK_{ATP}$  (Fig. 2C) currents decayed to  
 17% and 12% of their value at day 0, respectively. As for  $C_m$ , this decay became  
 35 significant ( $p < 0.05$ ) from day 2 on. The  $IK_1$  and  $IK_{ATP}$  currents decreased slowly  
 towards magnitudes not significantly different from 0 (Figs. 2B and 2C). Their  
 changes were fitted with a single exponential decay function (equation 1). The  
 time constants of decay were estimated to  $2.2 \pm 0.5$  and  $2.4 \pm 0.4$  days respectively,  
 they were not significantly different from that estimated to  $2.6 \pm 0.2$  days for  $C_m$   
 40 decay. Thus there was a close parallelism in the time course of disappearance of  
 $IK_1$  and  $IK_{ATP}$  with that of  $C_m$ .

*Relation between current magnitudes and cell capacitance*

5 The close parallelism of the changes in  $IK_1$  and  $IK_{ATP}$  with those of  $C_m$  suggests that these currents might be directly linked to the membrane pool that disappeared. To account for such a link, the hypothesis of a common determinant of their functional expression (genomic link) or of their maintenance in a functional state (e.g.: cytoskeletal rearrangement) might be invoked. Another  
 10 possibility is that the  $IK_1$  and  $IK_{ATP}$  channels are both mainly localized in the T-tubule membrane. In support of the hypothesis of localization of the  $IK_1$  current in cardiac T-tubules, the data of Mitcheson *et al.* (1996) show the  $IK_1$  current to decay in parallel with  $C_m$  in left ventricular rabbit myocytes in culture, despite a  
 15 4 fold faster time course of decay in  $C_m$  ( $\tau = 0.57$  day) than reported here (2.6 days). This discrepancy might be related to the fact that we used uncoated culture dishes whereas Mitcheson *et al.* used laminin attachment, which was reported to accelerate the loss of T-tubules (Delcarpio *et al.* 1989). A decrease of  $IK_1$  during primary culture is also observed in ventricular myocytes from adults rats (Wahler, 1992; Schackow *et al.*, 1995) whereas T-tubule density is decreased (Delcarpio  
 20 *et al.*, 1989). Thus changes in  $IK_1$  magnitude closely follow the amount of T-tubules in cardiomyocytes.

To our knowledge this is the first study to demonstrate a parallel decrease in  $IK_{ATP}$  with  $C_m$  in cardiomyocytes during cell culture. The hypothesis of  
 25 localization of  $IK_{ATP}$  channels in the T-tubules is supported by the observation that, in skeletal muscle, the  $K_{ATP}$  channel ligand P1075 preferentially binds to T-tubules (Dickinson *et al.*, 1997).

In line with the hypothesis of T-tubule localization of  $IK_1$  and  $IK_{ATP}$  channels,  
 30 the relation of cell current magnitudes of  $IK_1$  and  $IK_{ATP}$  to  $C_m$  values were analyzed by linear regression (see methods), which yielded regression coefficients of 0.93 and 0.98 respectively. The intercepts of the regression lines (Fig. 2D and E) with the  $C_m$  axis give an estimate of the capacitance of the membrane when tubular  $IK_1$  or  $IK_{ATP}$  currents have decayed to zero i.e.: 45 pF  
 35 and 40 pF respectively. These values are similar to the steady-state value of  $C_m$  (41 pF), that corresponds to the average capacitance of the external membrane of cells. The slope of the regression lines, that give an estimate of the current density at the tubular membrane, is 37 pA/pF for  $IK_1$  and 128 pA/pF for  $IK_{ATP}$ .



### *Time course of external Ba effects on the $IK_1$ current*

One consequence of the T-tubule localization of the  $IK_1$  conductance would be a diffusion delay in the access of an external blocker to the  $IK_1$  channels. This was tested using a local perfusion system (95% change in 50 ms) to apply 100  $\mu\text{M}$   $\text{BaCl}_2$  in external solution to a freshly dissociated cell. The cell was placed in front of the 300  $\mu\text{m}$  diameter outlet of a gravity-driven perfusion system (50  $\mu\text{l}/\text{min}$ ; Christé et al. 1999) that allowed switching to a new solution with a pure delay of 4 seconds, the time for 95% change being estimated to 50 ms from the change in the liquid junction potential at the tip of a patch pipette upon change in KCl concentration from 5.2 to 0.5 mM. The time course of onset and washout of the effect of  $\text{BaCl}_2$  was recorded at -120 mV (fig 3) and fitted by a simple model including diffusion in the T-tubules and binding of  $\text{Ba}^{++}$  to the  $IK_1$  channel (as described in the Materials and Methods).

In order to fit the data, parameters were initially set to values imposed by the actual data. The ratio  $k_2/k_1$  (i.e.: the apparent  $K_d$  of the  $IK_1$  channel for  $\text{Ba}^{++}$  ions) was set to 1.3  $\mu\text{M}$  at -120 mV, to account for the steady state fraction of  $IK_1$  remaining in the presence of 100  $\mu\text{M}$   $\text{BaCl}_2$ . This is near to a  $K_d$  of 3.25  $\mu\text{M}$  at -120 mV in guinea-pig ventricular myocytes (Hirano and Hiraoka, 1988). A detailed study of the mathematical system shows that the time course of onset of Ba effect mainly depends on  $k_1$  and  $\tau_T$  and that the time course of washout of the Ba effect is entirely defined by  $k_2$  and  $\tau_T$ . As a result, the system is constrained to a single set of final parameter values.

As may be readily noted from figure 3, the changes in the  $IK_1$  current upon  $\text{BaCl}_2$  application and wash followed a sigmoidal time course, which was reproduced when the time constant of diffusion ( $\tau_T$ ) was set to 300 ms. This value is in agreement with the time constants (250-270 ms) calculated by Levi *et al.* (1998) for the access of divalent cation blockers to L-type Ca channels that are situated in the T-tubules (Gao *et al.* 1997). Thus the time course of  $\text{BaCl}_2$  effects is consistent with the hypothesis that a large part of the  $IK_1$  conductance is located in the T-tubule membrane.

As a whole, the results of this study support the hypothesis that  $IK_1$  and  $IK_{\text{ATP}}$  channels in ventricular cardiomyocytes are preferentially localized in T-tubules. It is clear that a direct histological proof of the localization might be sought for, using appropriate specific antibodies against  $IK_1$  and  $K_{\text{ATP}}$  channels.

Physiological implications of such a localization of cardiac  $\text{K}^+$  channels deserve consideration. It is known that the Na-K pump, Na-Ca exchanger as well as Na channels are present in the T-tubular membrane and that the T-tubule is the major

site of E-C coupling in ventricular cells. In physiological conditions, accumulation of  $K^+$  ions in the T-tubule lumen (Freygang *et al.*, 1964) would tend to build up at high heart rates as a result of slow washout of  $K^+$  ions flowing outward through the  $IK_1$  channels. This might contribute to the supernormal  
5 excitability seen at high frequencies of stimulation, and also to shortening of action potential duration, as both a  $K^+$ -induced increase in  $K^+$  conductances and a depolarization of the T-tubule membrane are expected. Furthermore, through the interplay of luminal  $K^+$  elevation, Na-K pump stimulation, local changes in intracellular  $Na^+$  and in the Na-Ca exchange rate, the T-tubule may be the site of  
10 transient amplification phenomena taking part in the fine beat-to-beat tuning of E-C coupling.

In conditions of ischemia/hypoxia, further cumulative efflux of  $K^+$  ions through  $K_{ATP}$  channels (Yasui *et al.* 1993, Tourneur *et al.* 1994) may further depolarize the T-tubule membrane and cause triggered activities and eventually a loss of  
15 excitability. This may play a role in proarrhythmic as well as in protective effects (energy sparing due to impaired E-C coupling) known to occur in these pathological conditions.

The functional implications of the possible localization of  $K^+$  channels on the T-tubule function deserve further quantitative evaluation, both at the experimental  
20 and theoretical levels.

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- 15 ventricular cells. *Pflügers Arch* **422**: 371-379.

## Legends to Figures

**Figure 1:** *Superimposed whole cell current-voltage relations.*

Corrections were applied as described in Methods. After appropriate corrections, averages of whole cell current magnitudes were computed at each voltage during the descending ramp voltage protocol. **A:** average  $\pm$  S.E. of data from 10 cells at day 0 (trace a) and 9 cells at day 3 through 5 (trace b) in control Tyrode. **B:** Average at day 0 (trace a, n = 6) and at days 3 through 5 (trace b, n = 4), of maximal DNP-induced outward current.

**Figure 2:** *Analysis of the decay of  $C_m$ ,  $IK_1$  and  $IK_{ATP}$  with time in culture.*

The time dependent decay of  $C_m$  (A), and of the absolute magnitudes of the  $IK_1$  current (B) and the  $IK_{ATP}$  current (C) were fitted by a single exponential function with offset (continuous curves in A, B and C). Linear regression analysis was performed for  $IK_1$  versus  $C_m$  (D) and for  $IK_{ATP}$  versus  $C_m$  (E). The number of cells is given near each data point.

**Figure 3:** *Time course of block and unblock by external  $Ba^{++}$ .*

The  $IK_1$  current was continuously recorded at -120 mV. Plotted are the current values after after leak subtraction (corresponding to a 800 M $\Omega$  leakage membrane resistance) and normalization to maximal  $IK_1$  (circles in A, scattered trace in B).

A: response to the onset of  $Ba^{++}$  ions perfusion that started at the up arrow, B: response to the subsequent wash of  $Ba^{++}$  ions that started at the down arrow.

Note the difference in time scales between the two panels. Continuous thick lines in both panels show the best adjustment along the model presented in Materials and Methods. Parameter values were:  $k_2 = 0.257 \text{ sec}^{-1}$ ,  $k_1 = 1.97 \times 10^5 \text{ sec}^{-1} \cdot M^{-1}$ ,  $\tau_T = 300 \text{ ms}$ .

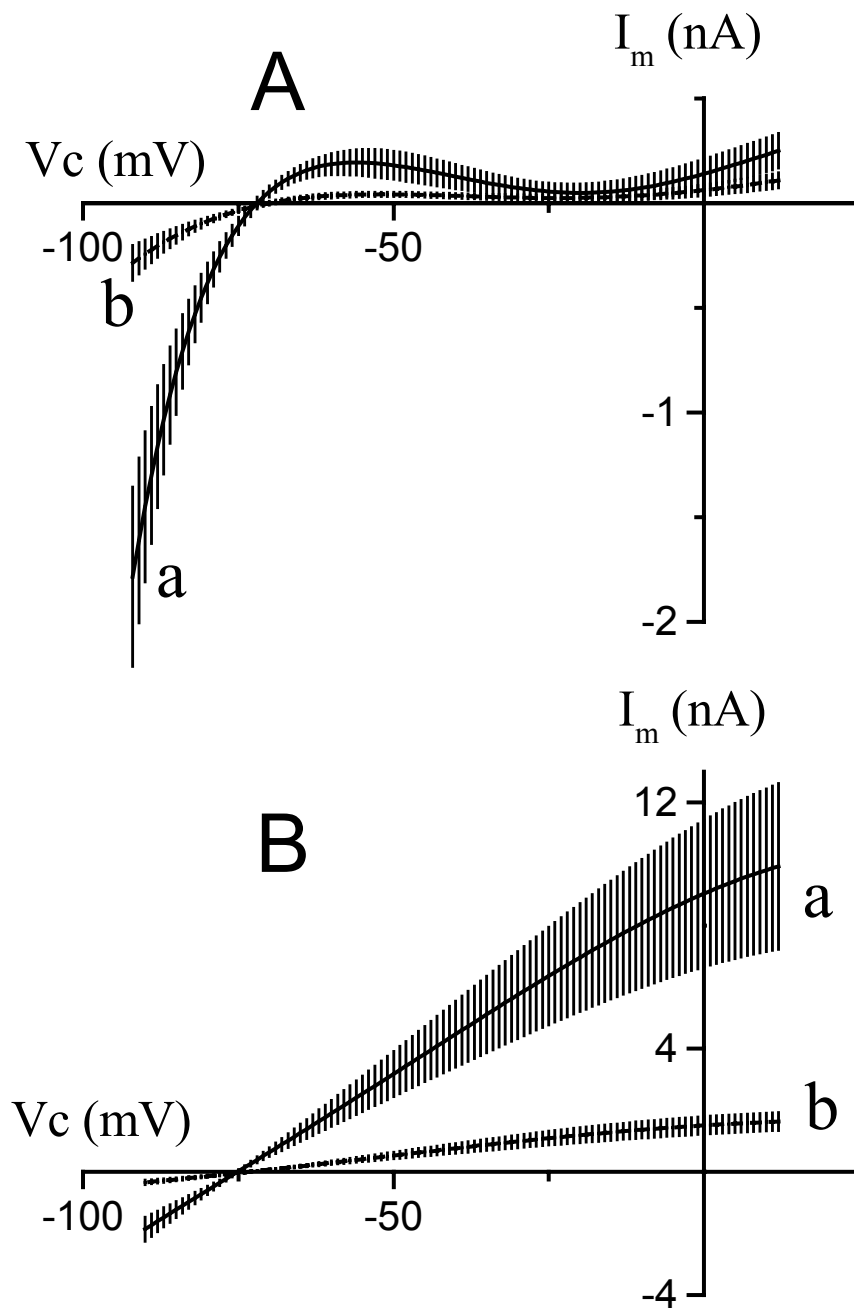


Figure 1

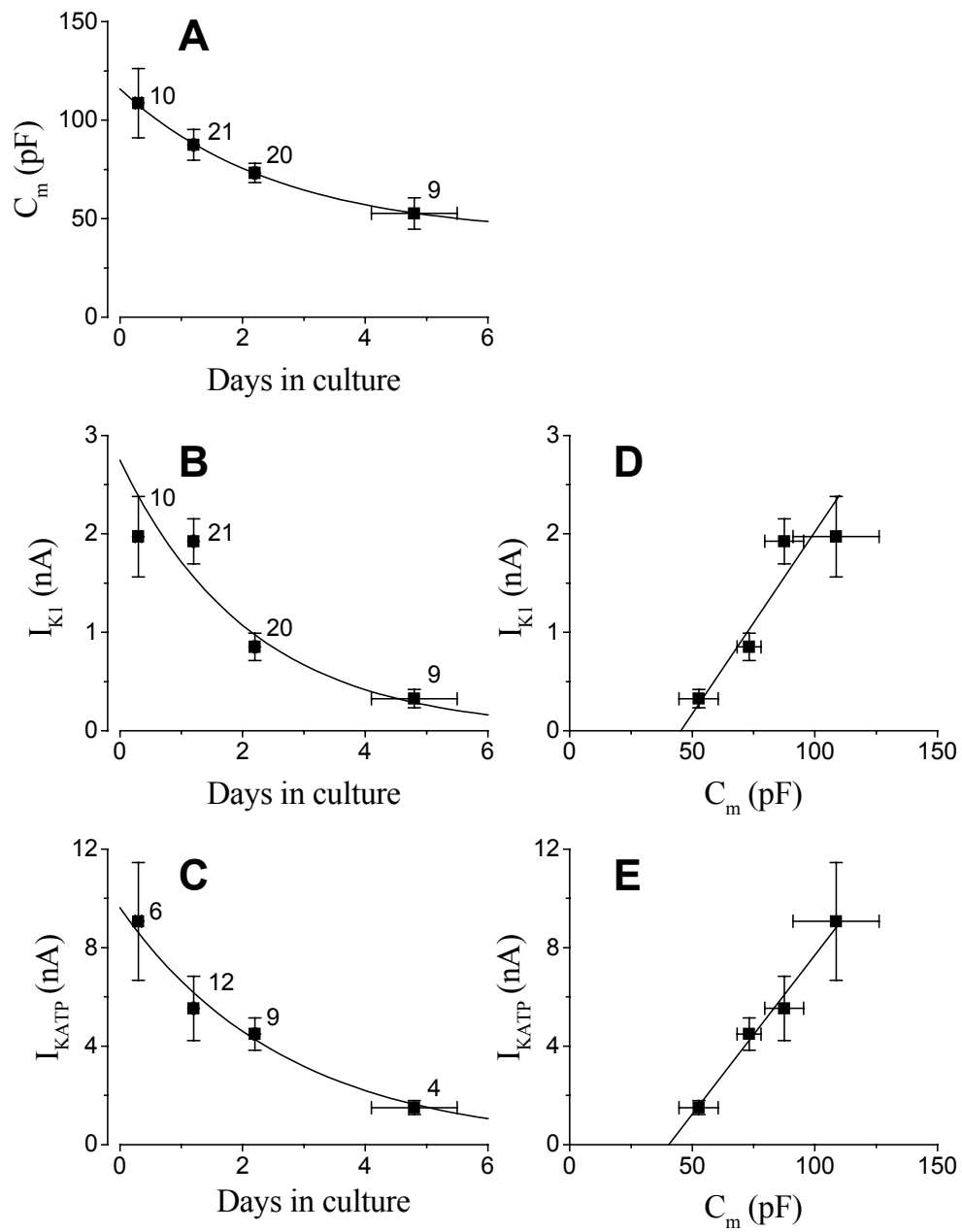


Figure 2

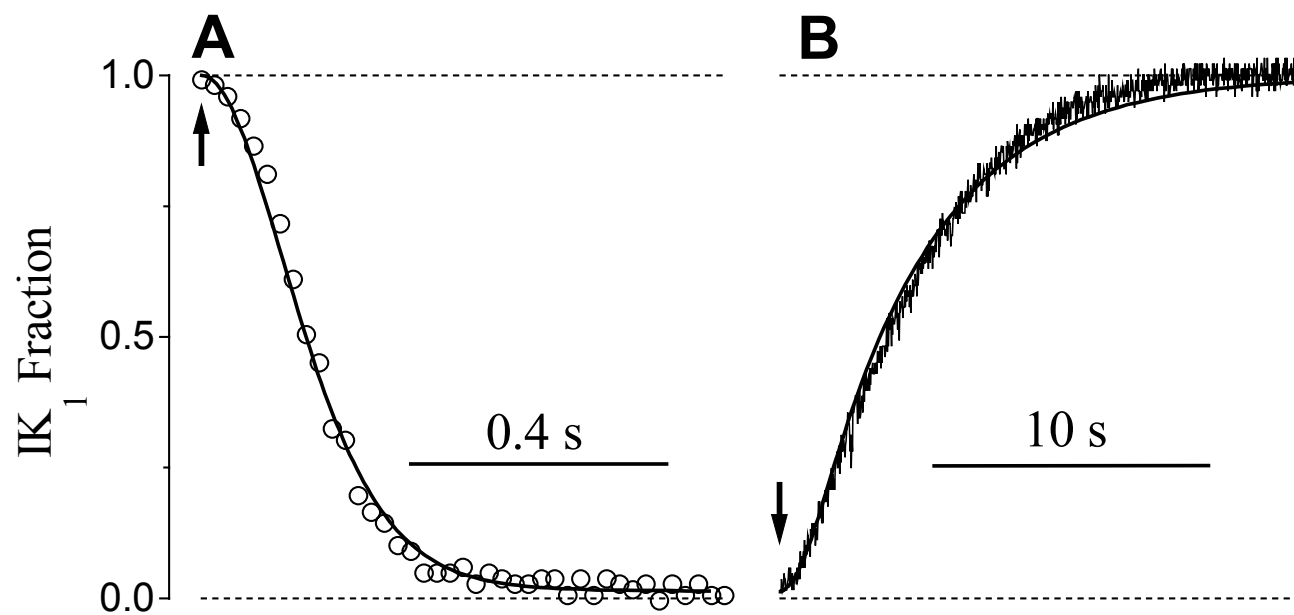


Figure 3