Localization of K(+) channels in the 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.

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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.

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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 (Cm) (Mitcheson *et al.* 1996). In this study, we have measured, in whole-cell voltage-clamped rabbit 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 Cm and the magnitudes of the background inward rectifier current (IK_1) and of the 2,4-dinitrophenol-induced ATP-sensitive potassium current (IK_{ATP}). Cm, IK_1 and IK_{ATP} all had 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±0.2, 2.2±0.5 and 2.4±0.4 days, respectively. Linear regressions of IK_1 and IK_{ATP} versus Cm 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 Cm. Furthermore, the time course of changes in

IK_1 when an external blocker (100 μM BaCl2) 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.

Taken together, these results could be explained by the localization of a major part of the IK_1 and IK_{ATP} currents of ventricular cardiomyocytes in the T-tubules. As a consequence, transient accumulation of K^+ ions in cardiac T-tubules may take place and modulate excitation-contraction coupling.

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 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 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 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 (Cm). Moreover, IK_1 changes upon rapid external application and 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.

Materials and Methods

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

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 $mg.kg^{-1}$ plus Ketamine 100 $mg.kg^{-1}$) 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 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; $MgCl_2$ 1.2; $CaCl_2$ 0.2; NaH_2PO_4 0.905; HEPES 10; glucose 10; pH was adjusted to 7.4 with NaOH. The pipette solution contained (mM): K-aspartate 115; KCl 5; $MgCl_2$ 7; K_2-ATP 4; Na_2-phosphocreatine 2; pyruvic acid 5; Na_2-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 $NaHCO_3$ 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 (Cm) and series resistance (Rs) 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 **I0**, the time constant **Tau** and the steady state current **Iss**. Cm was derived using the relation:

$$\mathbf{Cm = Tau * (1/Rs + 1/Rm)}$$

with

$$\mathbf{Rs = \Delta V/I0} \quad \text{and} \quad \mathbf{Rm = (\Delta V/Iss - \Delta V/I0)}.$$

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-1) 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, Rs-induced drop from command voltage and ramp-induced capacitance current as previously described (Christé et al. 1999). The main artefact was due to real Rs 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 IK_{ATP} 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 μM $BaCl_2$ (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 ± 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

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

where Y_0 is the value of Y(t) at time 0, τ 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:

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

where τ_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:

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

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 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). The current induced by 100 μM DNP (Fig 1B) was fully blocked by 10 μM 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.

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 time in culture. From an initial value of 109±18 pF (n=10) on day 0, it decayed to 53±8 pF (n=9) after about 4 days. The decay of Cm 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 ± 95% confidence interval), and the estimated steady-state level (41.4±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 Cm lost as computed from the present 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 Cm, this decay became 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±0.5 and 2.4±0.4 days respectively, they were not significantly different from that estimated to 2.6±0.2 days for Cm decay. Thus there was a close parallelism in the time course of disappearance of IK_1 and IK_{ATP} with that of Cm.

Relation between current magnitudes and cell capacitance

The close parallelism of the changes in IK_1 and IK_{ATP} with those of Cm 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 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 Cm in left ventricular rabbit myocytes in culture, despite a 4 fold faster time course of decay in Cm (τ = 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 *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 Cm in cardiomyocytes during cell culture. The hypothesis of 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, the relation of cell current magnitudes of IK_1 and IK_{ATP} to Cm 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 Cm 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 and 40 pF respectively. These values are similar to the steady-state value of Cm (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 µM $BaCl_2$ in external solution to a freshly dissociated cell. The cell was placed in front of the 300 µm diameter outlet of a gravity-driven perfusion system (50 µl/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 $BaCl_2$ was recorded at -120 mV (fig 3) and fitted by a simple model including diffusion in the T-tubules and binding of 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 k2/k1 (i.e.: the apparent Kd of the IK_1 channel for Ba^{++} ions) was set to 1.3 µM at -120 mV, to account for the steady state fraction of IK1 remaining in the presence of 100 µM BaCl2. This is near to a Kd of 3.25 µM 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 k1 and τ_T and that the time course of washout of the Ba effect is entirely defined by k2 and τ_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 $BaCl_2$ application and wash followed a sigmoidal time course, which was reproduced when the time constant of diffusion (τ_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 $BaCl_2$ 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_{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_{ATP} channels.

Physiological implications of such a localization of cardiac 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 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 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 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 and theoretical levels.

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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 ± 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 Cm, IK_1 and IK_{ATP} with time in culture*.
The time dependent decay of Cm (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 Cm (D) and for IK_{ATP} versus Cm (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 MΩ 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: $k_2 = 0.257\ sec^{-1}$, $k_1 = 1.97 \times 10^5\ sec^{-1}.M^{-1}$, $\tau_T = 300$ ms.

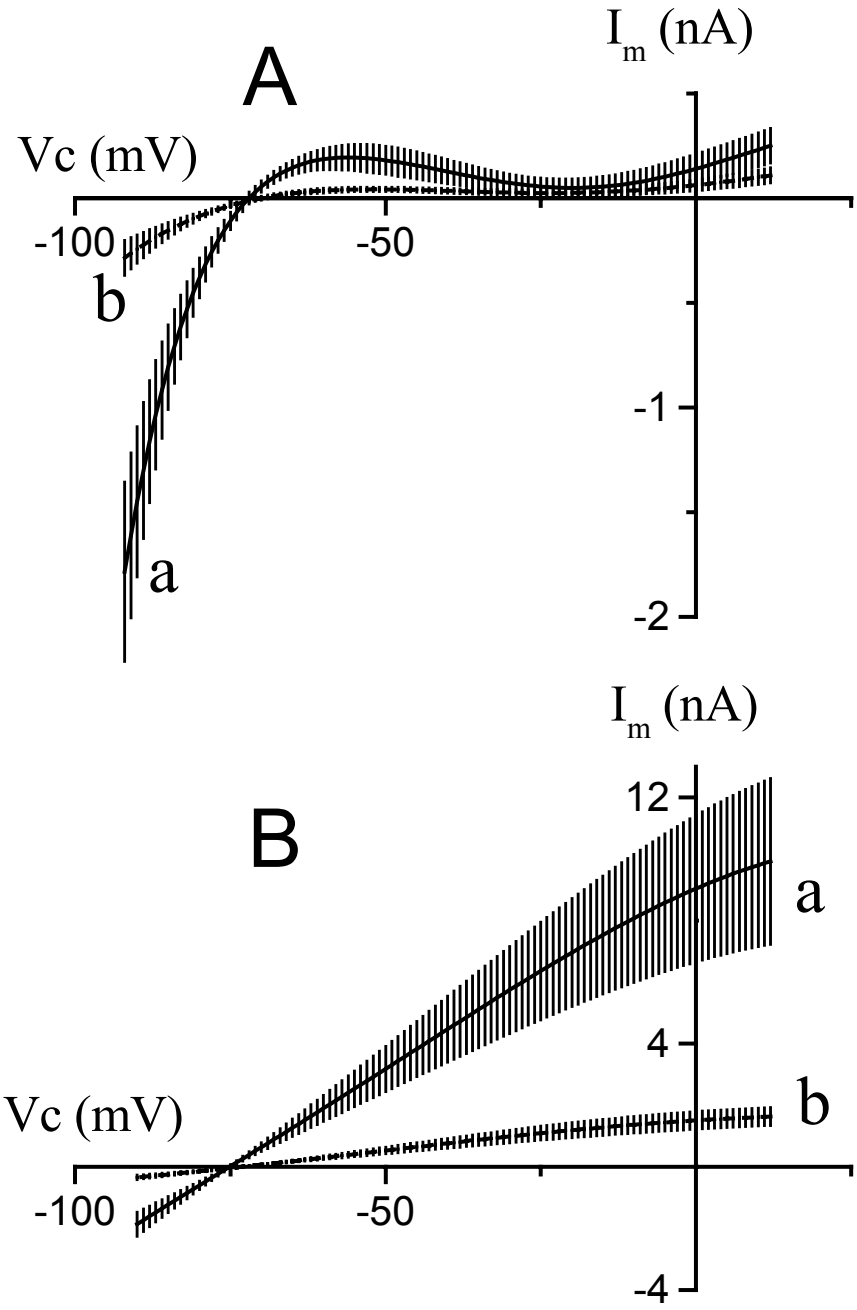

Figure 1

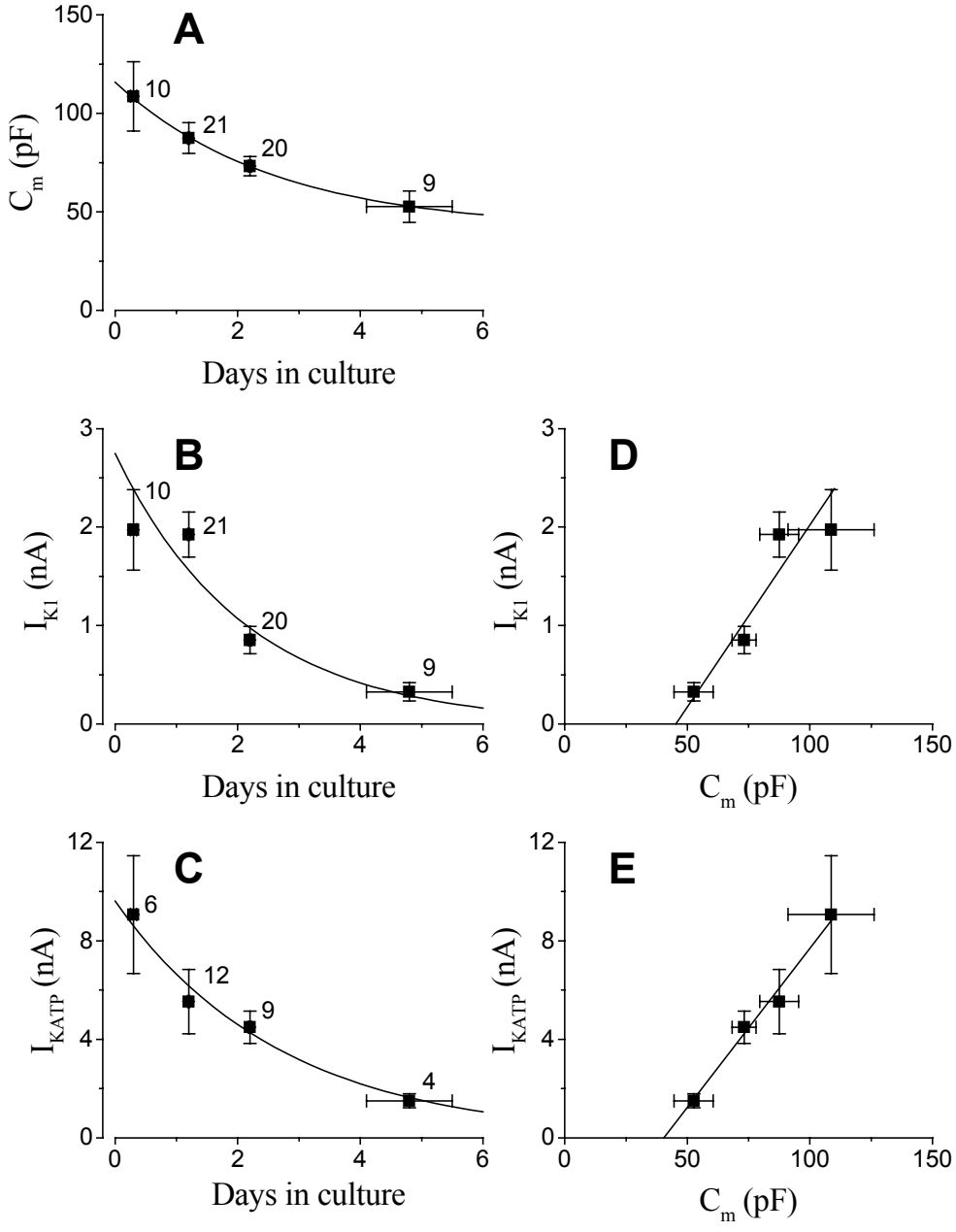

Figure 2

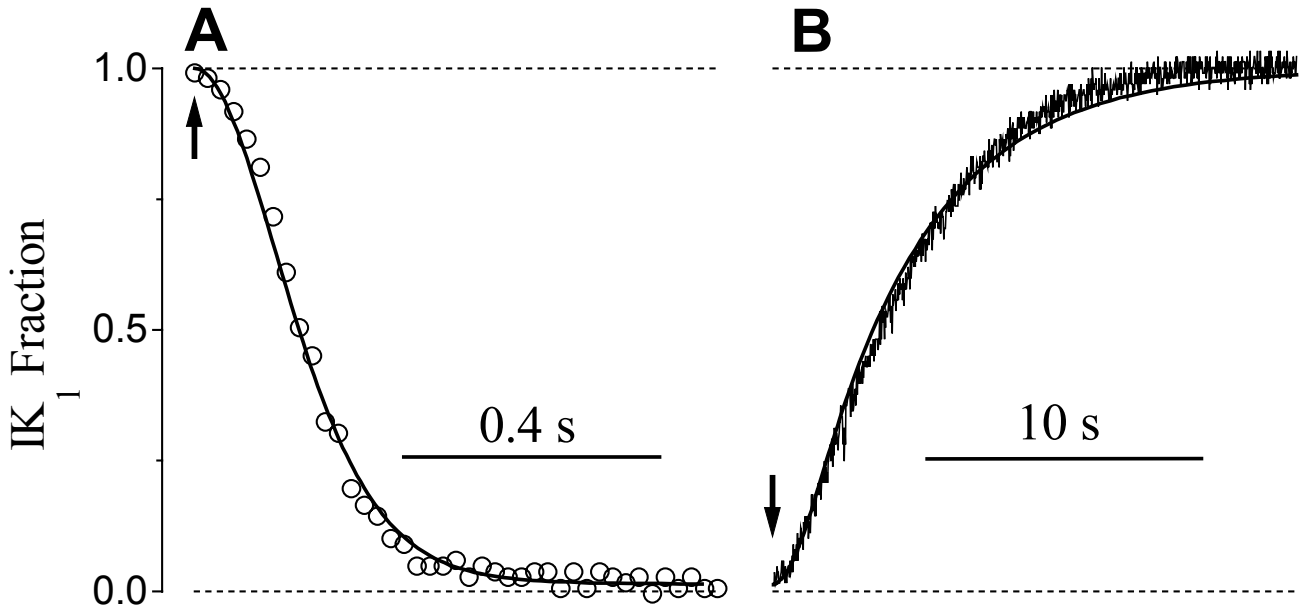

Figure 3