

Characterization of a hyperpolarization-activated current in dedifferentiated adult rat ventricular cells in primary culture

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(Received 25 June 1997; accepted after revision 11 September 1997)

1. The presence of a hyperpolarization-activated pacemaker (I_f)-like current was tested in dedifferentiated adult rat ventricular myocytes up to 12 days in primary culture with the whole-cell patch clamp technique.
2. An I_f -like current was found and characterized on freshly isolated and cultured ventricular cells. Both activation and density of the current varied in relation to the stage of dedifferentiation. The current was activated from -92.0 ± 2.5 and -63.0 ± 1.0 mV at the beginning (4-day-cultured cells) and end of the dedifferentiation process (12 days), respectively. Its density measured at -170 mV progressively increased from -2.34 ± 0.36 to -6.12 ± 0.64 pA pF⁻¹ between the two farthest stages of cellular remodelling. In freshly isolated cells the current was activated at -108.0 ± 1.5 mV and its current density measured at -170 mV was -1.97 ± 0.56 pA pF⁻¹.
3. The current was blocked by extracellular CsCl (3 mM) in a voltage-dependent manner. Modification of reversal potentials obtained at various values of $[K^+]_o$ (5.4 or 25 mM) and $[Na^+]_o$ (140 or 30 mM) suggests that the current was carried by both K⁺ and Na⁺ ions.
4. It is concluded that the hyperpolarization-activated inward current, recorded in freshly isolated and in cultured ventricular cells has characteristics similar to those of I_f . In adult rat ventricular cells it is activated in a non-physiological potential range, but can be elicited in a more physiological range when the cells are remodelled through a dedifferentiated way. It is suggested that such a current could be implicated in ventricular arrhythmias developed in pathological events.

Pacemaker activity is a property of specialized myocytes that are able to generate spontaneous action potentials (Irisawa, 1978). For these cells, an inward current flowing during diastole (phase 4 depolarization) is required. One of the ionic components involved in diastolic depolarization is the pacemaker current I_f (DiFrancesco, 1985; Irisawa, Brown & Giles, 1993). It is a time-dependent inward current activated by hyperpolarization and is caesium (Cs⁺) sensitive. The I_f channel is permeable to monovalent cations, allowing both Na⁺ and K⁺ ions to pass through. It has been observed in the sino-atrial (SA) node (Yanagihara & Irisawa, 1980), frog sinus venosus (Bois & Lenfant, 1990), atrioventricular node (Noma, Irisawa, Kokobun, Kotake, Nishimura & Watanabe, 1980), atrium (Earm, Shimoni & Spindler, 1983; Zhou & Lipsius, 1992) and Purkinje fibres (DiFrancesco, 1981). I_f has also been observed in the chick embryonic ventricle where it begins to activate at around -50 to -60 mV, in the same voltage range of phase 4 depolarization (Sperelakis, 1982; Satoh & Sperelakis, 1991). However, it disappears during cardiac development and this

disappearance parallels the reduction of the spontaneous pacemaker activity of these cells (Satoh & Sperelakis, 1991; Brochu, Clay & Shrier, 1992). In addition, I_f has recently been seen, in freshly isolated and cultured ventricular cells from newborn rats, to activate at around -70 mV (Robinson, Yu, Chang & Cohen, 1997). I_f has never been observed in adult mammalian ventricle in a physiological voltage range but it has been reported to exist in freshly isolated ventricular myocytes from adult guinea-pig, canine and rat hearts for potentials more negative than -120 mV (Yu, Chang & Cohen, 1993, 1995; Robinson *et al.* 1997). I_f has also been observed on ventricular cells isolated from hypertensive rats and from failing human hearts where it might favour the occurrence of spontaneous action potentials (Cerbai, Barbieri & Mugelli, 1994; Cerbai *et al.* 1997).

Adult cardiac ventricular myocytes have been shown to resume spontaneous contractility (Eppenberger, Hertig & Eppenberger-Eberhardt, 1994; Farès, Gomez & Potreau, 1996) when cultured in the 'redifferentiated model' (Jacobson

& Piper, 1986). In these conditions, the cells maintained in serum-containing medium for periods up to 4 weeks undergo dedifferentiation and redifferentiation processes with reactivation of the early fetal program of expression for several genes (Eppenberger *et al.* 1994). Also, we have recently reported that T-type calcium current, which has been shown to be present in rat ventricular myocytes at the neonatal stage but absent in adult cells, can be re-expressed when adult cells were dedifferentiated in culture (Farès *et al.* 1996).

Here we report that a time-dependent inward current is activated on hyperpolarization in dedifferentiated adult rat ventricular myocytes in culture. We provide evidence that this current has characteristics similar to those of I_f and that its voltage range for activation depends on the dedifferentiation stage of the cells.

METHODS

Cell isolation and culture

Ventricular myocytes were dissociated and cultured as previously described (Farès *et al.* 1996). Briefly, adult (250–300 g) male Wistar rats were injected with 1000 i.u. heparin i.p. (Choay; Sanofi, Gentilly, France) and anaesthetized with ether; the heart was quickly removed via thoracotomy and transferred to an ice-cold Tyrode solution. The aorta was cannulated and the heart mounted on a Langendorff apparatus and successively perfused (at 37 °C) with the following oxygenated solutions: 5 min with Tyrode solution to recover its spontaneous activity; 4 min with a nominally Ca^{2+} -free Tyrode solution, and about 20 min with the same solution supplemented with 0.05% collagenase (type II, Worthington), 0.06 mM CaCl_2 and 0.1% bovine serum albumin (BSA). When the heart was flaccid, it was rinsed with Kraft-Brühe (KB) medium (Isenberg & Klöckner, 1982) for 2 min. The ventricles were cut off, chopped into small pieces and gently stirred in KB medium. The isolated cells were filtered, maintained for 1 h in the KB medium and gradually resuspended in normal Tyrode solution. Part of these freshly isolated cells were kept in Tyrode solution at room temperature (20 ± 2 °C) for control experiments. For culture, other isolated cells were washed once in M199 medium (Gibco, Life Technologies) supplemented with 10^{-7} M insulin, 0.2% BSA, 10% fetal calf serum (Boehringer Mannheim) and 1% antibiotics (penicillin, 100 i.u. ml^{-1} ; streptomycin, 50 i.u. ml^{-1} ; Sigma), seeded into 35 mm Petri dishes pretreated with laminin ($20 \mu\text{g ml}^{-1}$; Sigma) and incubated for 30 min at 37 °C for plating. The culture medium was changed and supplemented with 10% fetal calf serum, and 10 μM cytosine 1- β -D arabinofuranoside (Sigma) to inhibit fibroblast proliferation. This medium was then renewed every 2 days.

Solutions

The Tyrode solution contained (mM): NaCl, 140; KCl, 5.4; CaCl_2 , 1.8; MgCl_2 , 1.8; Hepes, 10; glucose, 10; pH was adjusted to 7.35 with NaOH. To study the hyperpolarization-activated current, the extracellular control solution was modified to reduce the interference of components other than I_f , by adding to the Tyrode solution (mM): BaCl_2 , 8; MnCl_2 , 2; CdCl_2 , 0.2; 4-aminopyridine (Sigma), 0.5; plus tetrodotoxin (TTX; Sigma), 15 μM . For characterization of the current, the concentration of KCl was

increased to 25 mM or that of NaCl was reduced to 30 mM and replaced by equimolar *N*-methyl-D-glucamine (NMDG). The internal solution in the patch pipettes (2–4 M Ω) contained (mM): potassium aspartate, 130; MgCl_2 , 2; Na_2 -ATP, 2; CaCl_2 , 2; EGTA, 5; Na-GTP, 0.1; disodium phosphocreatine, 5; Hepes, 10; adjusted to pH 7.20 with KOH.

Experimental protocols

Voltage clamp experiments were performed at room temperature in the whole-cell configuration of the patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The electrical signal was recorded by a patch amplifier (RK 300, Biologic, Grenoble, France) and digitized through a conversion board (Labmaster TM40, Scientific Solutions, Solon, OH, USA). Programmed voltage clamp sequences and data acquisition were performed by a PC compatible computer (Epson, PC AX20, Nagano, Japan) using the pCLAMP software (version 5.5.1, Axon Instruments).

Membrane capacitance (C_m) was estimated from the capacitance transient elicited by a 10 mV depolarizing step from a holding potential (V_h) value of -90 mV and calculated according to the equation:

$$C_m = \tau_c (I_0 - I_\infty) / \Delta V_m,$$

where τ_c is the time constant of the membrane capacitance, I_0 is the initial current value, I_∞ is the amplitude of steady-state current and ΔV_m is the amplitude of voltage steps. No capacitance correction was used. Series resistance (R_s) ranged from 3 to 8 M Ω and was compensated in all cells by about 80%. I_f was elicited by hyperpolarization steps, varying from -60 to -170 mV from a holding potential of -40 mV.

Spontaneous activity was recorded in current clamp mode by means of the perforated patch method (Horn & Marty, 1988). For this, pipettes tips were filled with an antibiotic (Amphotericin B)-free solution containing (mM): KCl, 30; K_2SO_4 , 50; MgCl_2 , 8; Hepes, 10; pH, 7.2 with KOH. It should be noted that chloride ions were partly substituted by sulphate to minimize junction potential (Horn & Marty, 1988). The rest of the pipette was backfilled with the same solution containing Amphotericin-B (150 μM ; Sigma). Other specific protocols will be described in the text.

Data analysis

The amplitude of I_f was measured as the difference between the instantaneous current at the beginning of the hyperpolarizing step and the steady-state current recorded at the end of the step and normalized to C_m .

Specific conductance was determined as a function of membrane potential. At steady state the voltage dependence of I_f can be described by the equation:

$$I_f V = g_f V (V_m - V_{\text{rev}}),$$

where g_f is the conductance calculated at membrane potential V_m , and V_{rev} the reversal potential of the fully activated current derived from the analysis of tail currents of Fig. 3. Conductance–voltage (g_f – V) relations (Fig. 2*F*) were then obtained from the above equation as ratios between steady-state I – V curves I_f – V and V_m – V_{rev} . Conductance curves were fitted to the Boltzmann distribution:

$$g_f V = g_{\text{fmax}} V (1 / (1 + \exp((V_m - V_{0.5}) / S))),$$

where $g_{\text{fmax}} V$ is the maximum conductance, $V_{0.5}$ the half-maximal voltage of activation and S is the slope factor. This allowed

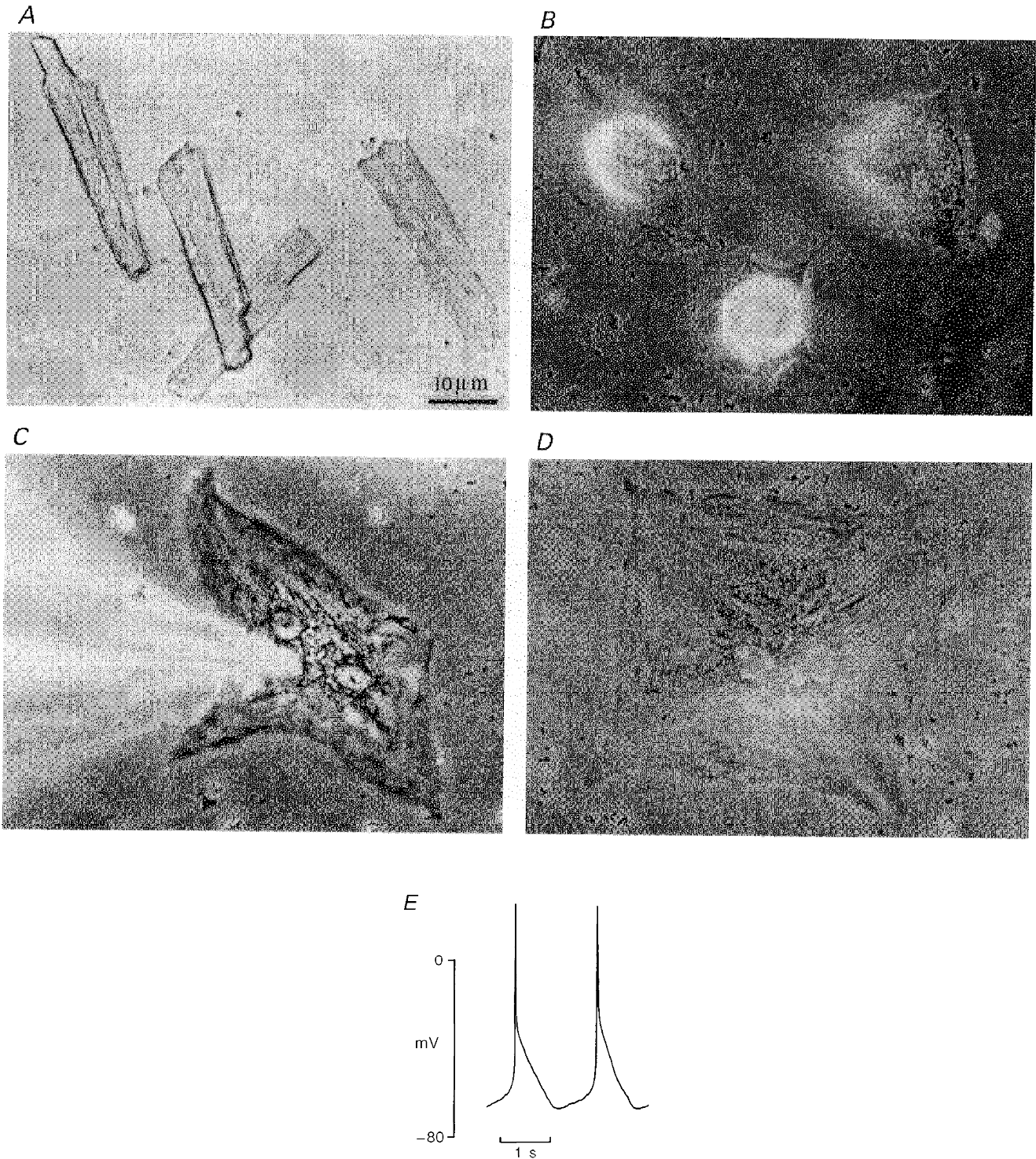


Figure 1. Changes in adult rat cardiomyocyte cytoarchitecture during long-term cell culture

Phase-contrast micrograph ($\times 400$) for freshly isolated cells (*A*) and for cells cultured for 4 (*B*), 8 (*C*) and 12 days (*D*) in M199 medium containing 10% fetal calf serum. The originally rod-shaped cells (*A*) undergo a morphological transition, first rounding and attaching to the substrate (4-day-cultured cells, *B*), then spreading and flattening (8 day cells, *C*) and finally displaying a large polymorphic configuration (12 day cells, *D*). At this stage, myocytes develop spontaneous contractile activity (*E*). Note in *C* the shadow of the patch pipette used for voltage clamp.

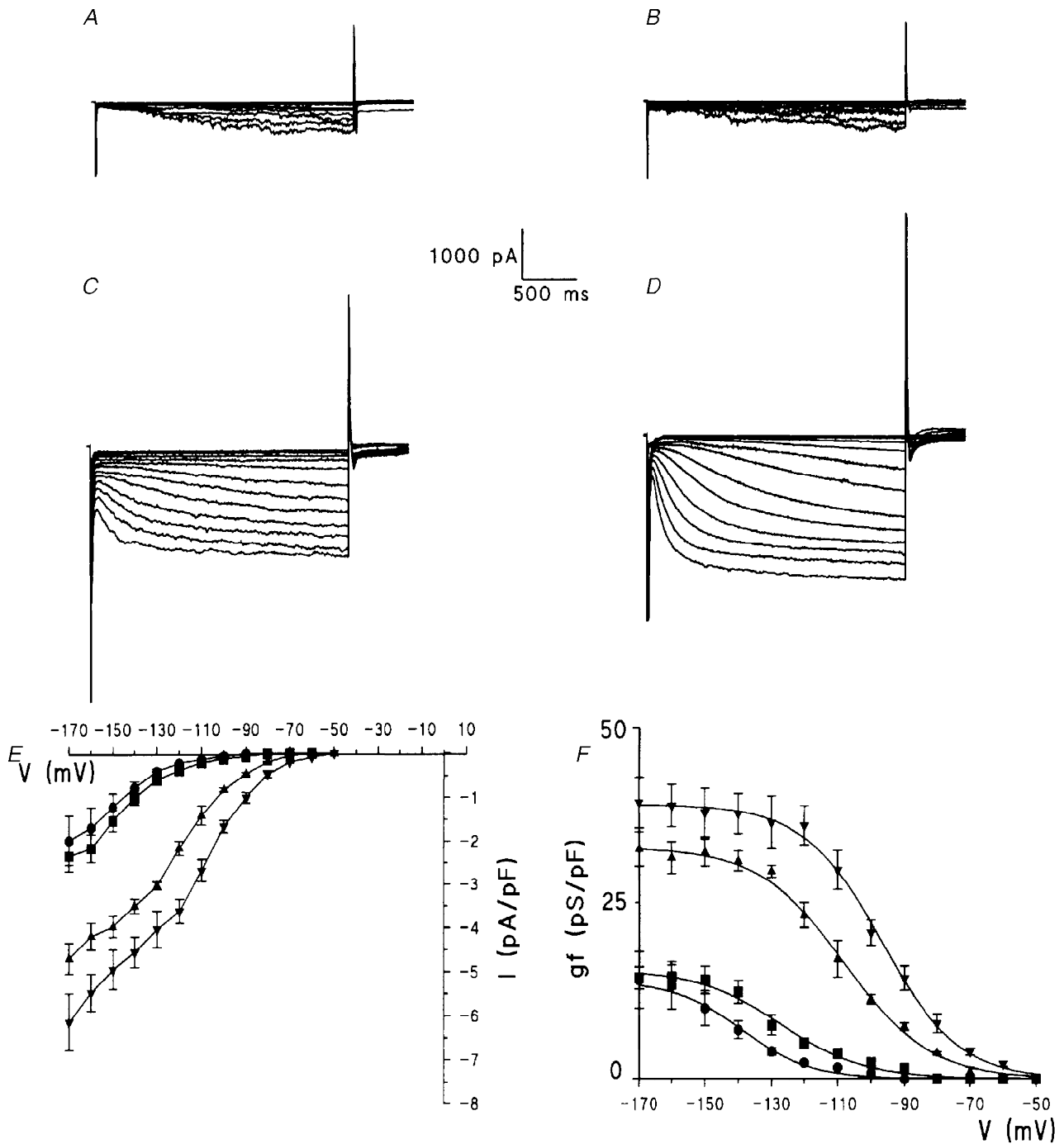


Figure 2. I_f characterization in dedifferentiated cardiac cells

Example of current traces for freshly isolated cells (A), 4-day- (B), 8-day- (C), and 12-day-cultured cells (D) recorded during 2.4 s pulses in the range -60 to -170 mV from a holding potential of -40 mV. E, superimposed $I-V$ curves of the hyperpolarizing-activated current recorded in various cell types. I_f was normalized with respect to membrane capacitance for freshly isolated cells ($n = 10$, ●) and for 4-day- ($n = 7$, ■), 8-day- ($n = 4$, ▲) and 12-day-cultured cells ($n = 7$, ▼). F, plot of mean conductance density (g_f) versus step potential calculated from current densities reported in E, with the equation described in Methods. Curves were fitted to data by a Boltzmann distribution. Data points are means \pm s.e.m.

estimation of the shifts of the voltage dependence of conductance measured as changes in $V_{0.5}$.

Results from multiple experiments are expressed as means \pm S.E.M. Statistical analysis was performed using analysis of variance, followed by the Newman–Keuls multiple range test for multiple experiments.

RESULTS

Correlation between cell dedifferentiation and the presence of a hyperpolarization-activated current

When cultured in the presence of M199 medium containing 10% fetal calf serum, adult rat cardiomyocytes undergo drastic remodelling (Eppenberger *et al.* 1994; Farès *et al.* 1996). Figure 1 shows that the dedifferentiation process of the freshly isolated cells (Fig. 1A) is characterized by gradual morphological transitions including initial loss of most traits of the cardiac phenotype followed by the flattening and spreading into a polymorphic cell shape. Several types were defined (types I, II and III): type I corresponds to the cells cultured for about 4 days, when the freshly isolated rod-shaped myocytes were attached to the substrate (Fig. 1A) and progress to a rounded shape (Fig. 1B); after 8 days of culture, most cells were largely flattened and considerably bigger with apparent multinucleation as shown in Fig. 1C and correspond to type II; after 12 days in culture, fully spread cells, defined as type III, exhibited large triangular extensions (Fig. 1D) and showed spontaneous activity (Fig. 1E). C_m increased in relation to the dedifferentiation process with significant increases in type II and type III cells (see Table 1). Furthermore, it should be noted that there was a transient decrease of C_m during the first days of culture, which could be explained by initial loss of cardiac phenotype and particularly by reduced T-tubular connections (Lipp, Hüser, Pott & Niggli, 1996).

In the whole-cell configuration (Fig. 2A–D), an inward current was activated by hyperpolarization in freshly isolated cells and in the three types of cultured cells. Figure 2E illustrates the current density–voltage relations. At -170 mV, the mean current density of freshly isolated cells was -1.97 ± 0.56 pA pF $^{-1}$ with a C_m of 157 ± 8 pF ($n = 8$). In type I cultured cells these values did not change significantly: -2.34 ± 0.36 pA pF $^{-1}$ and 153 ± 5 pF ($n = 7$), respectively. At the same potential, a significant increase of current density was observed in type II (-4.68 ± 0.35 pA pF $^{-1}$, $n = 4$) and in type III cells (-6.12 ± 0.64 pA pF $^{-1}$, $n = 7$) in parallel with a rise of C_m (263 ± 12 and 313 ± 12 pF, respectively). Figure 2F illustrates conductance density–voltage relations obtained from current density curves of Fig. 2E (see Methods). The potential values for activation threshold (T_a) and for half-conductance density ($V_{0.5}$) were largely negative in the freshly isolated cells and significantly decreased through the three types of cultured cells, leading to a shift of the

conductance density curves to less negative voltages, without a change in the slope factor (S) values. Moreover, maximum values of conductance density ($g_{fmax} V$) were significantly increased for type II and III cells (see Table 1).

In Fig. 3 the fully activated current density for freshly isolated cells (A) and for the three types of cultured cells (B–D) was evaluated over a large range of potential values ($+10$ to -70 mV) by measuring the tail current amplitudes after a hyperpolarizing pulse to -170 mV. The analysis of the curves shows that there is no significant change in the reversal potential (V_{rev}) values estimated from the intersection between the curves and the voltage axis. The maximum values of conductance density that correspond to the slopes of the curves (g_{fmax}), were similar to those obtained in Fig. 2, with the same increase in type II and III cultured cells (see Table 1).

Effect of extracellular caesium on the hyperpolarization-activated current

As reported in Purkinje fibres (DiFrancesco, 1982), in Purkinje cells (Callewaert, Carmeliet & Vereecke, 1984) and in SA node cells (DiFrancesco, Ferroni, Mazzanti & Tromba, 1986), Cs^+ is known to exert a voltage-dependent block of f-channels at potentials negative to the reversal potential for the pacemaker current. In cells cultured for 12 days (type III cells), the effect of Cs^+ was evaluated over a large range of potentials ($+10$ to -70 mV), by measuring the amplitude of tail currents following a hyperpolarizing step to -120 mV, which fully activates I_f (Fig. 4). Current traces recorded in control conditions (Fig. 4A) and after the addition of 3 mM CsCl show that the presence of Cs^+ results in an almost complete block of the inward current elicited by the step to -120 mV (Fig. 4B). In panel C, tail current amplitude, normalized to membrane capacitance, was plotted against the step voltage. In the presence of Cs^+ (filled circles), the negative region of the fully activated current density–voltage relation was characterized by a progressive reduction of the current showing a clear-cut voltage-dependent blockade. The reversal potential in the presence of Cs^+ was identical to that measured in control conditions (24.7 ± 0.1 mV, $n = 4$). A similar effect of Cs^+ was also observed on the current activated in the two other types of cultured cells and in freshly isolated cells (data not shown). The effect of Cs^+ on the hyperpolarization-activated current in cultured ventricular cells was qualitatively similar to that reported from Purkinje fibres (DiFrancesco, 1982), SA node cells (DiFrancesco *et al.* 1986), ventricular cells isolated from hearts of normotensive (Robinson *et al.* 1997) and hypertensive rats (Cerbai *et al.* 1994) and in ventricular myocytes isolated from failing human hearts (Cerbai *et al.* 1997). According to DiFrancesco (1982) the effect of Cs^+ on I_f is voltage dependent and the current is completely blocked at voltages more negative than -120 mV. In these conditions the residual inward current observed in the presence of Cs^+ between -30 and -70 mV (Fig. 4C) is probably a part of I_f not fully suppressed at this voltage range.

Ionic selectivity of the hyperpolarization-activated current

I_f is dependent on external potassium and sodium in Purkinje fibres and SA node cells (Callewaert *et al.* 1984; DiFrancesco *et al.* 1986). In order to investigate whether potassium and sodium influence the hyperpolarization-activated current of cultured ventricular cells, their concentrations were modified in the perfusion solution.

Effect of increasing the external potassium concentration

The measurement of tail current amplitude was used again to evaluate the reversal potential of I_f at two extracellular potassium concentrations. Current traces in Fig. 5 were obtained in type III cells superfused with Tyrode solution containing either 5.4 mM (Fig. 5A) or 25 mM $[K^+]_o$ (Fig. 5B). The most obvious effect caused by the augmentation in

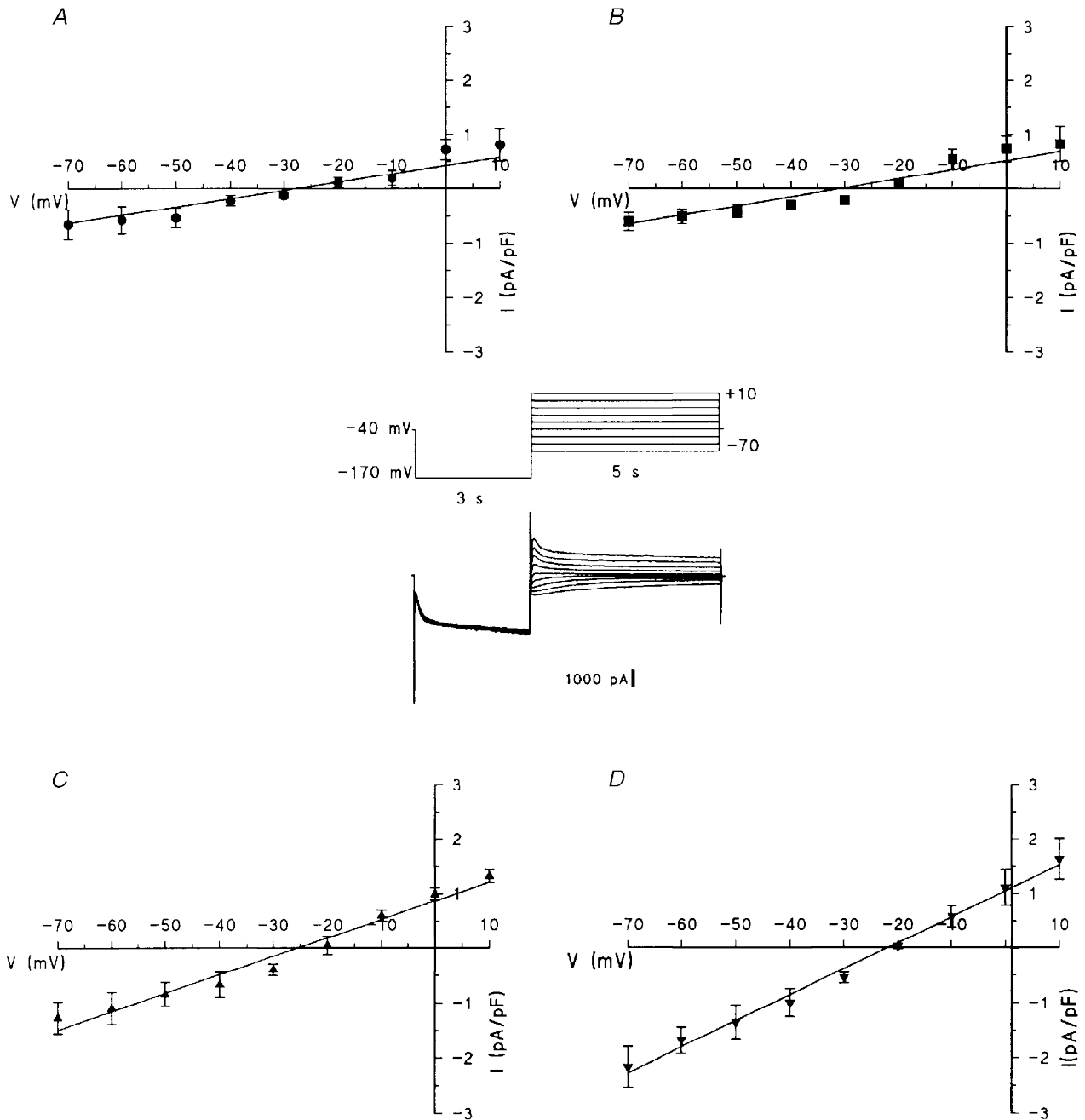


Figure 3. Fully activated I - V relations of I_f

The tail current amplitude was measured after a hyperpolarizing pulse of -170 mV, normalized to membrane capacitance and plotted *versus* voltage values from $+10$ to -70 mV, for freshly isolated cells (A , $n=7$), 4-day- (B , $n=6$), 8-day- (C , $n=6$) and 12-day-cultured cells (D , $n=10$). Data points are means \pm s.e.m. Inset, an example of current traces recorded from a 12-day-cultured cell.

Table 1. Comparative data measured for *I_f* in freshly isolated cells and in the three types of cultured cells

	Freshly isolated cells	4-day-cultured cells (Type I)	8-day-cultured cells (Type II)	12-day-cultured cells (Type III)
<i>C_m</i> (pF)	157 ± 8 (8)	153 ± 5 (7)	263 ± 12*† (4)	313 ± 12*†† (7)
<i>T_a</i> (mV)	-108.0 ± 1.5 (10)	-92.0 ± 2.5* (7)	-75.0 ± 2.0*† (4)	-63.0 ± 1.0*†† (7)
<i>V_{rev}</i> (mV)	-29.0 ± 0.1 (7)	-31.2 ± 0.1 (6)	-27.6 ± 0.1 (6)	-23.0 ± 0.1 (10)
<i>g_{fmax}</i> <i>V</i> (pS pF ⁻¹)	13.90 ± 0.40 (10)	15.45 ± 0.77 (6)	33.02 ± 0.51*† (4)	39.15 ± 0.44*†† (7)
<i>g_{fmax}</i> (pS pF ⁻¹)	15.00 ± 0.15 (7)	16.58 ± 0.18 (6)	33.57 ± 0.21*† (6)	47.36 ± 0.15*†† (10)
<i>V_{0.5}</i> (mV)	-138.8 ± 0.9 (10)	-127.7 ± 2.0* (7)	-108.1 ± 0.9*† (4)	-96.9 ± 0.7*†† (7)
<i>S</i>	10.31 ± 0.80	12.60 ± 1.50	12.47 ± 0.71	11.66 ± 0.60

C_m, cell membrane capacitance; *T_a*, potential for activation threshold; *V_{rev}*, reversal potential; *g_{fmax}* *V*, maximum conductance obtained from Boltzmann fitting (Fig. 2); *g_{fmax}*, slope of the fully activated current (Fig. 3); *V_{0.5}*, half-maximum voltage; and *S*, slope factor. Values are means ± s.e.m. * *P* < 0.01 compared with freshly isolated cells; † *P* < 0.01 compared with type I cells; †† *P* < 0.01 compared with type II cells.

[K⁺]_o was a marked increase in *I_f* amplitude. Tail current amplitudes, obtained in four different cells superfused with 5.4 mM [K⁺]_o and normalized to membrane capacitance, are plotted as a function of tail step potential in the *I-V* curve of Fig. 5C (open squares). The best fit line through data points gave a linear relation that intersected the *X*-axis at -22.8 ± 0.1 mV with a *g_{fmax}* of 44.50 ± 3.00 pS pF⁻¹. In the presence of 25 mM [K⁺]_o, the reversal potential was

-13.8 ± 0.3 mV and *g_{fmax}* was 90.20 ± 3.50 pS pF⁻¹. Such a shift of the reversal potential is comparable to that reported for *I_f* in Purkinje fibres (DiFrancesco, 1982) and SA node cells (DiFrancesco *et al.* 1986) exposed to a similar increase in extracellular K⁺. The rise in conductance as a consequence of increasing [K⁺]_o is also typical for *I_f* in primary cardiac pacemaker cells (DiFrancesco *et al.* 1986; Frace, Maruoka & Noma, 1992).

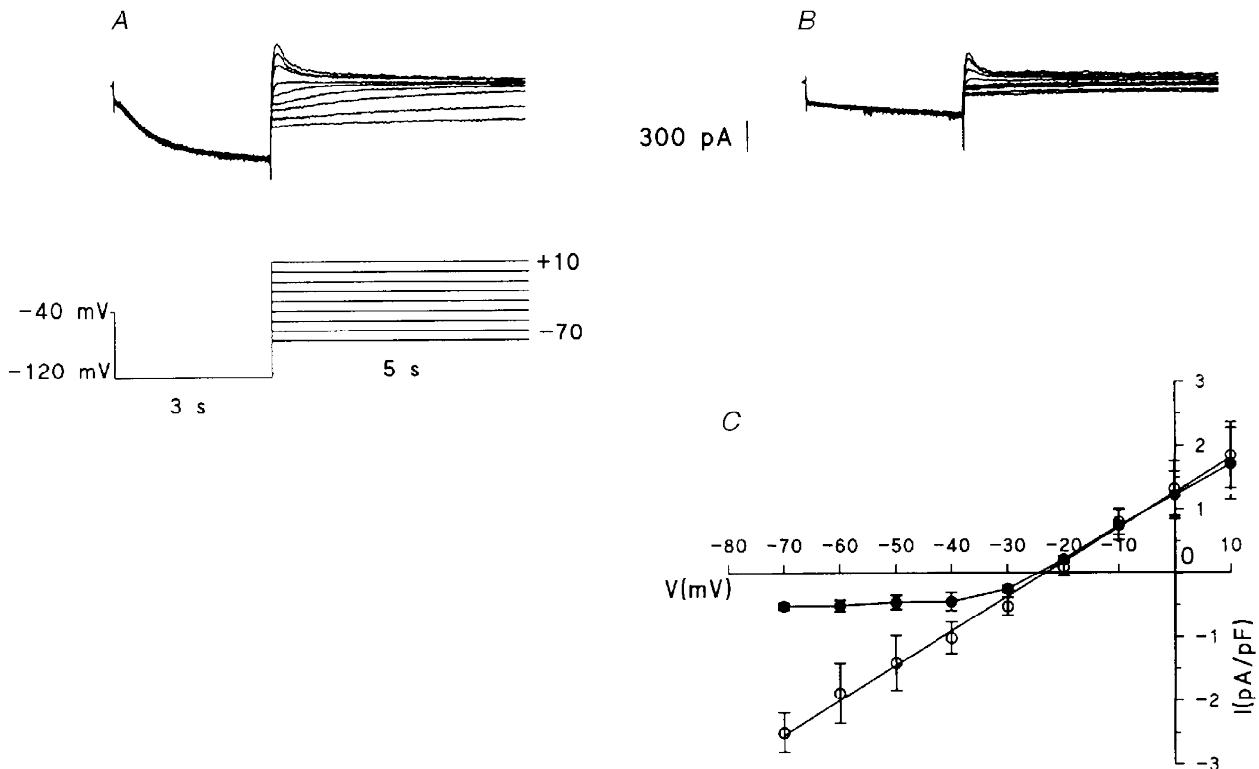


Figure 4. Effect of caesium on the hyperpolarizing-activated current

Current traces are recorded in a 12-day-cultured myocyte (*C_m* = 305 pF) in control conditions (A) and after the addition of 3 mM CsCl (B) with the voltage protocol reported in A. C, fully activated *I-V* curves for tail current amplitude, normalized to membrane capacitance and plotted against the step voltage in control (○) and in the presence of 3 mM CsCl (●). Data points are means ± s.e.m., *n* = 4.

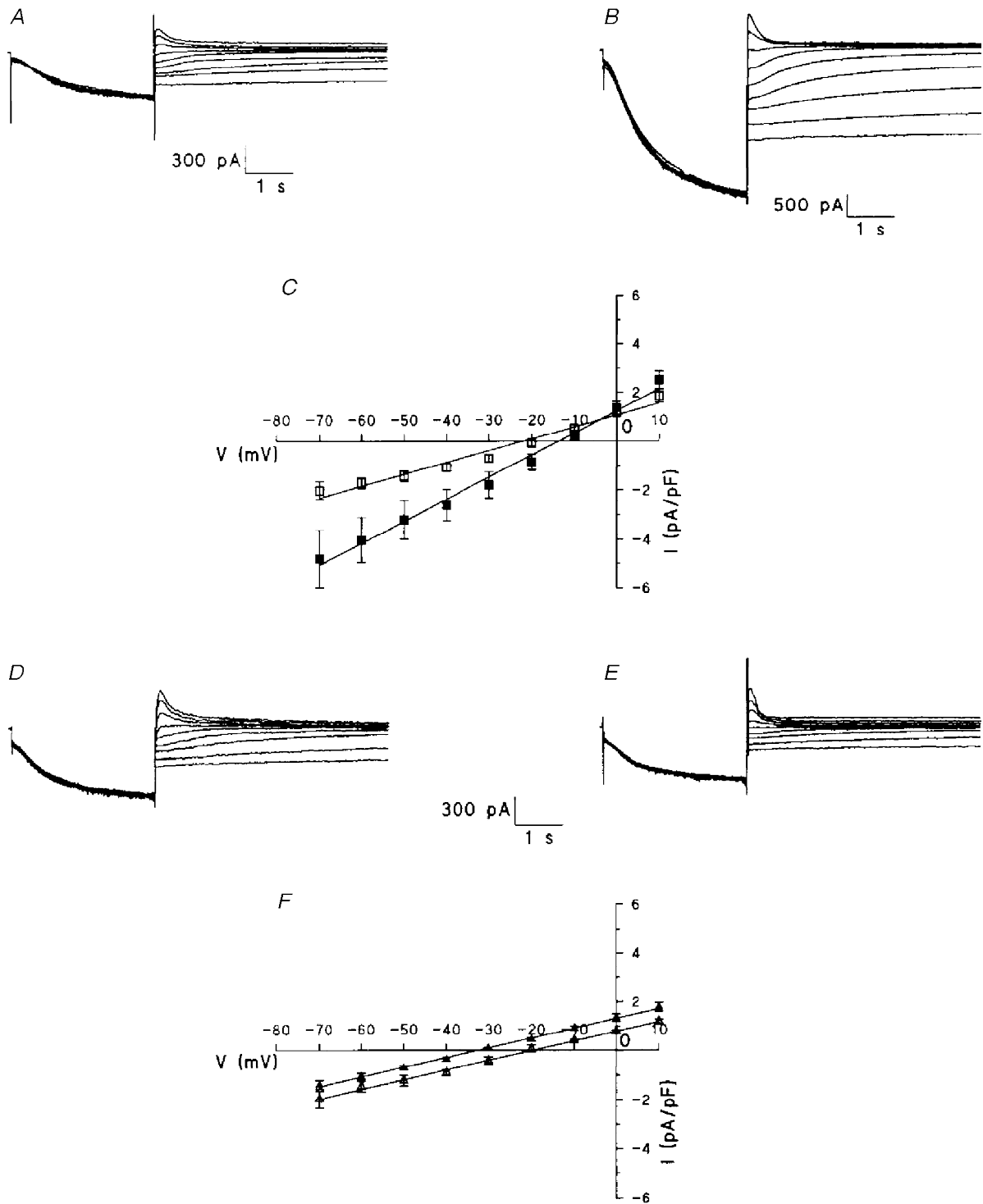


Figure 5. Effects of changes in external potassium and sodium concentrations on the fully activated current

A–C, effect of increasing K⁺ concentration from 5.4 (A and C: □) to 25 mM (B and C: ■). Note the positive shift of the reversal potential and the increase of the curve slope in C (n = 4). D–E, effect of decreasing Na⁺ concentration from 140 (D and F: △) to 30 mM (E and F: ▲). Note the negative shift of the reversal potential without changing the slope in F (n = 6). In panels C and F, data points are means ± s.e.m.

On the other hand, freshly isolated cells exhibit a similar shift of V_{rev} to less negative values (shift of V_{rev} of 11 ± 5 mV, $n = 3$) when increasing $[K^+]_o$ from 5.4 to 25 mM. These data are compatible with those obtained by Yu *et al.* (1995) on adult cells isolated from guinea-pig hearts.

Effect of reducing the external sodium concentration

Figure 5D–F reports the effect of reducing the external sodium concentration from 140 mM (control conditions) to 30 mM on the hyperpolarization-activated current in type III cultured cells with the above protocol. The fully activated current in control (Fig. 5D) was decreased after reducing the external sodium concentration to 30 mM (Fig. 5E). The fully activated current density curves show that data points obtained from six cells in control and 30 mM $[Na^+]_o$, respectively, were fitted by linear relations (Fig. 5F). In both conditions, g_{fmax} was comparable (39.40 ± 0.90 and 39.70 ± 0.70 pS pF⁻¹ in 140 and 30 mM $[Na^+]_o$, respectively) suggesting that the conductance of the channel is not affected by reducing extracellular sodium. However, the reversal potential obtained in control (-20.5 ± 0.2 mV) is significantly shifted toward hyperpolarization (-33.6 ± 0.4 mV) in reduced sodium conditions. The shift is comparable to that reported for I_f in SA node cells (DiFrancesco *et al.* 1986) or frog sinus venosus cells (Bois & Lenfant, 1990) exposed to a similar reduction in extracellular sodium. Figure 5F also shows faster current kinetics in low sodium solution. This observation needs further experiments to explain this.

DISCUSSION

The present results indicate that the time-dependent inward current exists in dedifferentiated adult rat ventricular myocytes in primary culture. In type III cultured cells, this current has the electrophysiological characteristics of I_f present in pacemaker cardiac cells. It is activated upon hyperpolarization, is caesium sensitive and is selective for Na^+ and K^+ . A similar inward current activated by hyperpolarizations can be observed in less dedifferentiated cells in culture and even in freshly isolated cells. However, this current is activated at more negative voltages and its density is smaller than this of I_f recorded in type III cells. Such a current previously reported in freshly isolated adult rat ventricular cells (Robinson *et al.* 1997), but not observed in all cells (Cerbai, Barbieri & Mugelli, 1996), will need further characterization to be clearly considered as I_f .

Analysis of the conductance density curves shows that the electrophysiological characteristics change with the time of culture: a shift of potential activation threshold towards less negative values (-92 mV for type I to -63 mV for type III cells) and an increase of the conductance density from type I to type III cells (by about 150%). The present shift may be due to the increasing activities of cAMP which directly acts on f-channels (DiFrancesco & Tortora, 1991) and/or the increasing activities of protein kinases and

decreasing activities of phosphatases, enhancing the phosphorylation of channels (Yu *et al.* 1995). It is interesting to note that a shift of the voltage dependence of I_f towards large negative values inversely occurs from newborn to adult rat ventricle myocytes (Robinson *et al.* 1997). This negative shift may be due to a gene switch, a developmentally regulated translational or post-translational alteration in the channel, or the appearance (or disappearance) of an associated protein.

The rise of conductance should be explained by an over-expression of the f-channel gene leading to an augmentation in the number of functional channels. Such an upregulation during culture of adult ventricular cardiomyocytes was already reported for several genes: β -myosin heavy chain (Eppenberger-Eberhardt, Flamme, Kurer & Eppenberger, 1990; Dubus, Rappaport, Barrieux, Lompré, Schwartz & Samuel, 1993), atrial natriuretic factor (Clark, Rudnick, LaPres, Andersen & LaPointe, 1993; Swynghedauw, 1993), T-type calcium channels (Farès *et al.* 1996). This process is generally correlated to an increase in cell size and to cytoskeletal changes (Eppenberger *et al.* 1994).

Finally, the present results show that type III cultured cells exhibit pacemaker activity with a maximum diastolic potential around -60 mV and a marked depolarization phase (Fig. 1E). As reported in a previous study (Farès *et al.* 1996), the resting membrane potential measured in control Tyrode solution significantly decreases throughout the culture in serum-containing medium from -77 ± 3 mV ($n = 20$) for freshly isolated cells to -65 ± 4 mV ($n = 21$) in 8-day-old cultured cells and -63 ± 2 mV ($n = 12$) for the maximum diastolic potential measured in spontaneously beating cells after 13 days in culture (type III). Such values can be compared to those recorded in primary pacemaker cells (Irisawa, 1978).

A recent report demonstrated that cardiomyocytes in biopsies from patients with chronic myocardial ischaemia and marked anterior wall motion abnormalities showed signs of dedifferentiation rather than necrotic properties (Ausma *et al.* 1995). Moreover, it has been suggested that I_f contributes to the increased propensity for ventricular arrhythmias of hypertrophied rat heart and of human failing heart (Cerbai *et al.* 1994, 1997). In these conditions, the present model of dedifferentiated adult ventricular myocytes in primary culture seems to offer an opportunity to investigate the potential role of I_f in the genesis of ventricular arrhythmias.

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Acknowledgements

This work was supported by grants from CNRS, University of Poitiers and Fondation Langlois.

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