Functional expression and regulation of the hyperpolarization activated non-selective cation current in embryonic stem cellderived cardiomyocytes

N. Abi-gerges, G. Ji, Z. Lu, R. Fischmeister, J. Hescheler, B. Fleischmann

Functional expression and regulation of the hyperpolarization activated non-selective cation current in embryonic stem cell-derived cardiomyocytes

N. Abi-Gerges *, G. J. Ji †, Z. J. Lu †, R. Fischmeister *, J. Hescheler † and B. K. Fleischmann †

†*Institute of Neurophysiology, University of Cologne, D-50931 Cologne, Germany and* **Laboratoire de Cardiologie Cellulaire & Moléculaire, INSERM U-446, Faculty of Pharmacy, University of Paris-Sud, F-92296 Châtenay-Malabry, France*

(Received 30 April 1999; accepted after revision 19 November 1999)

1. The biophysical and pharmacological characteristics of the hyperpolarization activated non-selective cation current (I_f) were recorded using whole-cell voltage clamp in embryonic stem (ES) cell-derived cardiomyocytes at different stages of development.

2. The cation current was detected in a large percentage (65%) of early stage (EDS, differentiated for 7 + 3–4 days) cells at a current density of $11{\cdot}4 \pm 0{\cdot}6$ pA pF^{-1} ($n = 47$). In late stage (LDS, differentiated for 7 + 9–12 days) cells the percentage of cells expressing I_f decreased (45%), but I_f densities ($15{\cdot}5 \pm 0{\cdot}9$ pA pF^{-1}, $n = 20$) were increased.

3. The muscarinic agonist carbachol (CCh, 1 μM) depressed basal I_f in EDS cells by $45{\cdot}7 \pm 6{\cdot}5$%, $n = 5$) and was without effect in LDS cardiomyocytes ($n = 4$). The β-adrenoceptor agonist isoprenaline (ISO, 1 μM) stimulated I_f in LDS cells by $33 \pm 5{\cdot}2$% ($n = 6$) but not in EDS cells ($n = 5$).

4. Cell infusion with the catalytic subunit of the cAMP-dependent protein kinase (PKA, 7 μM) stimulated I_f in EDS cells by $37{\cdot}0 \pm 2{\cdot}9$%, ($n = 4$), but subsequent superfusion of 8-bromo-cAMP (200 μM) was without effect. Intracellular perfusion of LDS cardiomyocytes with the highly selective peptide inhibitor of PKA (PKI, 20 μM) completely inhibited the stimulation of the L-type Ca^{2+} current ($I_{Ca,L}$) as well as of I_f by ISO (1 μM).

5. Extracellular superfusion with phosphodiesterase (PDE) inhibitors – IBMX, a non-selective antagonist, Erythro-9-(2-hydoxy-3-nonyl)adenine (EHNA), a PDE2 antagonist and rolipram, a PDE4 antagonist – resulted in stimulation of $I_{Ca,L}$ and I_f in EDS cells. By contrast, milrinone and cilostamide, two PDE3 antagonists, stimulated $I_{Ca,L,}$ but not I_f.

6. The present work demonstrates that I_f is functionally expressed during early cardiomyogenesis. Similar to $I_{Ca,L}$, I_f is regulated during embryonic development by phosphorylation via PKA. In contrast to $I_{Ca,L}$, I_f is not regulated by PDE3 suggesting different localization of these ion channels with respect to PDE3.

A key feature of the heart is its spontaneous electrical activity, which is under the regulatory control of the autonomic nervous system. The automaticity of the sinus node is due to the diastolic depolarization and the hyperpolarization-activated non-selective cation current I_f appears to be involved in its generation (DiFrancesco, 1993). The biophysical characteristics of I_f comprise time-dependent activation kinetics, a linear current–voltage (I–V) relationship and voltage-dependent block upon extracellular application of Cs^+. Recently, I_f has been cloned and shown to display similar structural characteristics to other voltage-gated ion channels (Gauss *et al.* 1998; Ludwig *et al.* 1998; Santoro *et al.* 1998). As also reported for other cyclic nucleotide-gated ion channels, the carboxyl terminal region of I_f contains a consensus sequence site for cAMP-dependent protein kinases (Ludwig *et al.* 1998).

The non-selective cation current I_f has been detected in cells of the cardiac conductive system of different species such as frog sinus venosus (Bois & Lenfant, 1990), rabbit sino-atrial node (Yanagihara & Irisawa, 1980), rabbit atrioventricular node (DiFrancesco *et al.* 1980), cat atrium (Zhou & Lipsius, 1992*a*) and rabbit Purkinje fibres (DiFrancesco & Ferroni, 1983). In addition, I_f is present during embryonic and perinatal development since the current has been observed in chick embryonic ventricle (Satoh & Sperelakis, 1991), in freshly isolated and cultured ventricular cells from newborn

rats (Robinson *et al.* 1997) as well as in dedifferentiated adult rat ventricular cells from primary cultures (Fares *et al.* 1998). In contrast to early postnatal development, I_f has been detected in adult mammalian ventricle (Yu *et al.* 1993, 1995), but only upon applying strongly hyperpolarizing test pulses (negative to −120 mV).

It is well known that the autonomic nervous system regulates the chronotropy of the heart and that I_f is regulated by sympathetic and parasympathetic neurotransmitters. Indeed, the β-adrenoceptor agonist isoprenaline (ISO) stimulates I_f by shifting the activation curve to more positive voltages whereas the vagal neurotransmitter acetylcholine (ACh) inhibits I_f by shifting the activation curve to more negative potentials (DiFrancesco & Tromba, 1988). I_f regulation clearly involves the modulation of adenylyl cyclase activity (Lindemann & Watanabe, 1990; Pappano, 1990) and both the direct binding of cAMP to f-channels (DiFrancesco & Tortora, 1991) and the phosphorylation of the channels via cAMP-dependent protein kinase A (PKA) (Chang *et al.* 1991; Yu *et al.* 1995) contribute to the overall effect on I_f.

Since pathologically altered cardiac myocytes re-express early embryonic genes (Vikstrom & Leinwand, 1996) and I_f has been detected in ventricular cardiomyocytes of hypertensive rats (Cerbai *et al.* 1996) as well as recently in human failing heart (Cerbai *et al.* 1997; Hoppe & Beuckelmann, 1998), we examined the expression and modulation of I_f during early cardiomyogenesis. We have shown earlier that the regulation of the L-type Ca^{2+} channel current ($I_{Ca,L}$) during early stages of cardiomyogenesis differs strongly from that known for terminally differentiated cardiomyocytes. At these early stages intrinsic adenylyl cyclase and phosphodiesterase (PDE) activities are high, whereas β-adrenoceptors are not yet functionally coupled (Ji *et al.* 1999; Maltsev *et al.* 1999). Due to the known difficulties of harvesting murine cardiomyocytes from the early murine embryo prior to E11.5 (Davies *et al.* 1996), we have used in the present study murine embryonic stem (ES) cell-derived cardiomyocytes, known to recapitulate cardiomyogenesis *in vitro* (Wobus *et al.* 1991; Maltsev *et al.* 1994; Hescheler *et al.* 1997).

We demonstrate that I_f is expressed early during murine cardiomyogenesis and displays similar biophysical characteristics to those reported for terminally differentiated cardiomyocytes. We further show that during embryonic development I_f is modulated by phosphorylation and that I_f and $I_{Ca,L}$ are differentially regulated by PDEs.

METHODS

Embryonic stem cell preparation

Murine ES cells of the line D3 were cultured and differentiated into spontaneously beating cardiomyocytes as previously described (Wobus *et al.* 1991; Maltsev *et al.* 1994). Briefly, ES cells differentiated within spheroidal aggregates (embryoid bodies) in hanging drops for 2 days. The embryoid bodies were thereafter transferred into suspension for 5 days and finally plated for different periods of time. The EDS cells were kept in suspension for 7 days and thereafter plated for 3–4 days (7 + 3–4) prior to dissociation. The IDS cells were kept for 7 days in suspension and plated for 5–8 days (7 + 5–8). The LDS cells were also kept in suspension for 7 days and plated for 9–12 days (7 + 9–12) in 24 micro-well plates. Single cardiomyocytes were isolated from clusters of spontaneously beating areas by a modification of the procedure described by Isenberg & Klöckner (1982). Beating areas of 15 to 20 embryoid bodies were isolated with a sterile microscalpel and collected in low Ca^{2+} solution containing (mM): 120 NaCl, 5·4 KCl, 5 $MgSO_4$, 5 sodium pyruvate, 20 glucose, 20 taurine, 10 Hepes (pH 6·9 with NaOH). The tissue was then incubated in enzyme medium (1 mg ml^{-1} collagenase B, Boehringer, Mannheim, FRG; 30 μM $CaCl_2$) for 20 min at 37 °C. Tissue fragments were transferred to a medium containing (mM): 85 KCl, 30 K_2HPO_4, 5 $MgSO_4$, 1 EGTA, 2 Na_2ATP, 5 sodium pyruvate, 5 creatine, 20 taurine, 20 glucose, pH 7·2, where they were kept at room temperature for 1 h, and then resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco, Eggenstein, FRG) complemented with 20 % fetal calf serum. Isolated cells were plated onto sterile, gelatine-coated glass cover slips and kept in an incubator for 24–48 h. Spontaneously contracting myocytes could be observed within 12 h after cell preparation.

Electrophysiology

For patch clamp recordings only spontaneously beating, single cardiomyocytes were selected, using the whole-cell configuration of the patch-clamp technique (Hamill *et al.* 1981). The cells were held in the voltage-clamp mode using an Axopatch 200-A (Axon Instruments, Foster City, USA) or an EPC-9 (Heka, Lambrecht, FRG) amplifier. For I_f measurements, cardiomyocytes were clamped at a holding potential of −35 mV for 200 ms to obtain inactivation of Na^+ currents and hyperpolarizing pulses to −110 mV (2–5 s duration) were used. For recording of the steady-state activation curves, hyperpolarizing voltage steps (2 s) from −40 to −160 mV in 10 mV intervals were applied (holding potential −35 mV) and tail currents measured at 15 mV. The current–voltage (I–V) relationship for I_f was determined by measuring the tail currents (2 s) 15 ms after voltage steps to potentials ranging from −120 to 50 mV in 10 mV step intervals following a 2 s hyperpolarizing voltage step to −115 mV (holding potential (V_h) −35 mV). The regulation of I_f was investigated by applying hyperpolarizing pulses to −110 mV (holding potential −35 mV). Due to the slow activation kinetics of I_f, voltage steps were applied at low frequency (maximum rate, 0·1 Hz). The protocol for the simultaneous recording of $I_{Ca,L}$ and I_f consisted of depolarizing steps from a holding potential of −50 mV to 0 mV (200 ms duration, activating preferentially for $I_{Ca,L}$) followed by a hyperpolarization to −110 mV for 2 s for the recording of I_f. The hyperpolarizing step to −110 mV was followed by a depolarization to −35 mV for 50 ms to inactivate $I_{Ca,L}$ prior to returning to a holding potential of −50 mV. Cell membrane capacity was determined on-line using the ISO2 (MFK, Frankfurt, FRG) or Pulse (Heka) acquisition software program. Data were acquired at a sampling rate of 10 kHz, filtered at 1 kHz, stored on hard disk and analysed off-line using the ISO2 or the Pulsefit (Heka) analysis software package. The glass cover slips containing the cells were placed into a temperature-controlled (37 °C) recording chamber and perfused continuously with extracellular solution by gravity at a rate of 1 ml min^{-1}. Substances were applied by exchanging the solution in the chamber; a 90 % volume exchange was achieved within approximately 20 s. Patch pipettes (2–4 MΩ resistance) were pulled from Clark borosilicate glass (Electromedical Instruments, Reading, UK) using a Zeitz puller (DMZ, Munich,

FRG). The solutions used for voltage-clamp recordings had the following composition (mM): internal solution for measurement of I_f: NaCl 10, potassium aspartate 130, Na_2ATP 2, Na_2GTP 0·1, $MgCl_2$ 2, EGTA 1, Hepes 10, pH 7·2 (adjusted with KOH). TEA (10 mM) was added to the I_f internal solution for the simultaneous recording of I_f and $I_{Ca,L}$. External solution for measurement of I_f (mM): NaCl 140, NaOH 2·3, $MgCl_2$ 1, KCl 5·4, $CaCl_2$ 1·8, Hepes 5, glucose 10, $CdCl_2$ 0·5, $BaCl_2$ 1,4-aminopyridine 2, pH 7·4 (adjusted with NaOH). In the experiments showing a simultaneous recording of both $I_{Ca,L}$ and I_f, 0·5 mM $CdCl_2$ was omitted from the I_f external solution.

Analysis of data

The amplitude of I_f was measured as the difference between the instantaneous current at the beginning of the voltage step and the steady-state current recorded at the end of the voltage step (Cerbai *et al.* 1994). Currents were normalized to cell membrane capacity where indicated. For the analysis of steady-state activation curves, the amplitude of the tail currents was determined as the difference current between peak and sustained currents and Boltzmann distributions fitted to the normalized values. For *I–V* relations, the amplitude and the reversal potential of currents were analysed from the tail currents measured 15 ms after application of the voltage step in order to avoid contamination by Na^+ or capacitance currents (Hoppe & Beuckelmann, 1998). In the time course diagrams the time point '0' indicates the beginning of recordings shortly after establishment of the whole-cell configuration. When Cs^+ or carbachol (CCh) were applied, the instantaneous current amplitude in the presence of Cs^+ or CCh was taken as the reference value for the estimation of I_f. For the estimation of I_f densities data from all EDS cells used in these experiments were included, whereas in order to increase the number of IDS and LDS cells, data from experiments not reported here were included. Averaged data are expressed as means ± S.E.M. Statistical analysis was performed using Student's paired and unpaired t tests and a P value of $< 0·05$ was considered significant. Substances were applied only after establishment of stable current amplitudes.

Reagents

The catalytic subunit of PKA was obtained from Promega (Heidelberg, FRG) and the protein kinase A inhibitor (PKI, fragment 6–22 amide) was purchased from Sigma (Deisenhofen, FRG). PKI was dissolved in intracellular solution and stored frozen at −20 °C. The aliquots were thawed immediately prior to use and diluted with pipette solution to the desired concentration. Cilostamide was obtained from Calbiochem (Meudon, France). All other substances were purchased from Sigma (Deisenhofen, FRG) and freshly prepared by dissolving in extracellular solution.

RESULTS

Biophysical and pharmacological characteristics of I_f in EDS cells

The whole-cell configuration of the patch-clamp technique was used for the recording of I_f in isolated ES cell-derived cardiomyocytes of different developmental stages. Figure 1*A* illustrates a representative experiment where the steady-state current–activation curve of I_f was recorded in a spontaneously contracting EDS cardiomyocyte. It can be seen that the threshold of activation of I_f was close to −50 mV. However, a precise determination could not be made since applying much longer voltage steps was not tolerated by the cells. The activation curve was determined by measuring tail currents at +15 mV upon fully activating I_f by applying hyperpolarizing voltage steps from −30 to −160 mV (left panel). Peak amplitudes of tail currents were measured in three representative EDS and/or IDS cardiomyocytes, normalized and fitted with a Boltzman function yielding a V_h of $-98·1 \pm 1·1$ mV (Fig. 1*A*, right panel). Next we determined the current–voltage (*I–V*) relationship by measuring the tail currents at different potentials after a prepulse (2 s) to −115 mV (Fig. 1*B*). As can be seen in Fig. 1*B* (right panel) the *I–V* relation is linear with a reversal potential close to −30 mV. Thus, the slow activation kinetics (left panel), the relatively linear *I–V* relationship with an average reversal potential of $-30·9 \pm 1·1$ mV ($n = 4$) and the activation threshold close to −50 mV were similar to those reported for I_f in sino-atrial node cells or Purkinje fibres from adult hearts (for review see DiFrancesco, 1993). It was reported for Purkinje (Callewaert *et al.* 1984) and sino-atrial (SA) node cells (DiFrancesco, 1986) that Cs^+ exerts a voltage-dependent block on f-channels at potentials negative to the reversal potential of I_f. Similarly, extracellular application of Cs^+ (2 mM) in ES cell-derived cardiomyocytes strongly inhibited I_f (Fig. 1*C*, $n = 10$). The time course of a representative experiment, where each data point represents the amplitude of I_f evoked by a voltage step to −110 mV (holding potential −35 mV, see original traces in the inset, left panel) demonstrates an almost complete block of the inward current by Cs^+ (Fig. 1*C*, left panel) and the reversibility of the block upon washout in an EDS cell. The right panel of Fig. 1*C* shows the *I–V* curve recorded in another cardiomyocyte where the voltage dependence of the inhibition of I_f by Cs^+ was tested. In the presence of Cs^+ (filled squares) I_f is blocked at potentials negative to the reversal potential, whereas at potentials positive to the reversal potential the amplitude of I_f evoked by depolarizing voltage steps is unaltered. Our results demonstrate that the overall biophysical and pharmacological characteristics of I_f in ES cell-derived cardiomyocytes are similar to those reported in adult cardiomyocytes.

Figure 2*A* shows the percentage of cells expressing I_f at different stages of cardiomyogenesis. I_f was detected in a large percentage (65%, $n = 18$) of EDS cells (7 + 3–4) days, whereas at the IDS (7 + 5–8) day stage, I_f was detected in fewer ES cell-derived cardiomyocytes (50%, $n = 10$). Moreover, the percentage of cells expressing I_f was even less (45%, $n = 8$) in LDS cells (7 + 9–12) days. The I_f densities were $11·4 \pm 0·6$ pA pF^{-1} ($n = 47$) and $11·9 \pm 1·7$ pA pF^{-1} ($n = 11$) in EDS and IDS cells, respectively. LDS cells displayed a significantly higher current density of $15·5 \pm 0·9$ pA pF^{-1} ($n = 20$, $P < 0·05$) as compared with EDS and IDS cells (Fig. 2*B*).

Effects of β-adrenergic and muscarinic agonists on I_f in EDS and LDS cells

It is well established that β-adrenergic and muscarinic agonists stimulate and inhibit I_f, respectively (DiFrancesco & Tromba, 1987; Zhou & Lipsius, 1992*b*; DiFrancesco, 1995*a*; Renaudon *et al.* 1997). Since the steady-state activation

curves yielded a relative negative V_h and modulation of I_f is known to work through a shift in the activation curve on the voltage axis, I_f was first evoked by depolarizing steps to −110 mV. In line with our previous findings on the development-dependent establishment of the regulation of $I_{Ca,L}$ (Maltsev *et al.* 1999; Ji *et al.* 1999), CCh did not depress basal I_f in LDS cells (Fig. 3*B*, I_f control density: 12·7 ± 1·9 pA pF^{-1}; I_f density in presence of CCh: 12·5 ± 1·9 pA pF^{-1}, $n = 4$) whereas ISO did not stimulate I_f in EDS cells (Fig. 4*A*, control I_f density: 11·9 ± 2 pA pF^{-1};

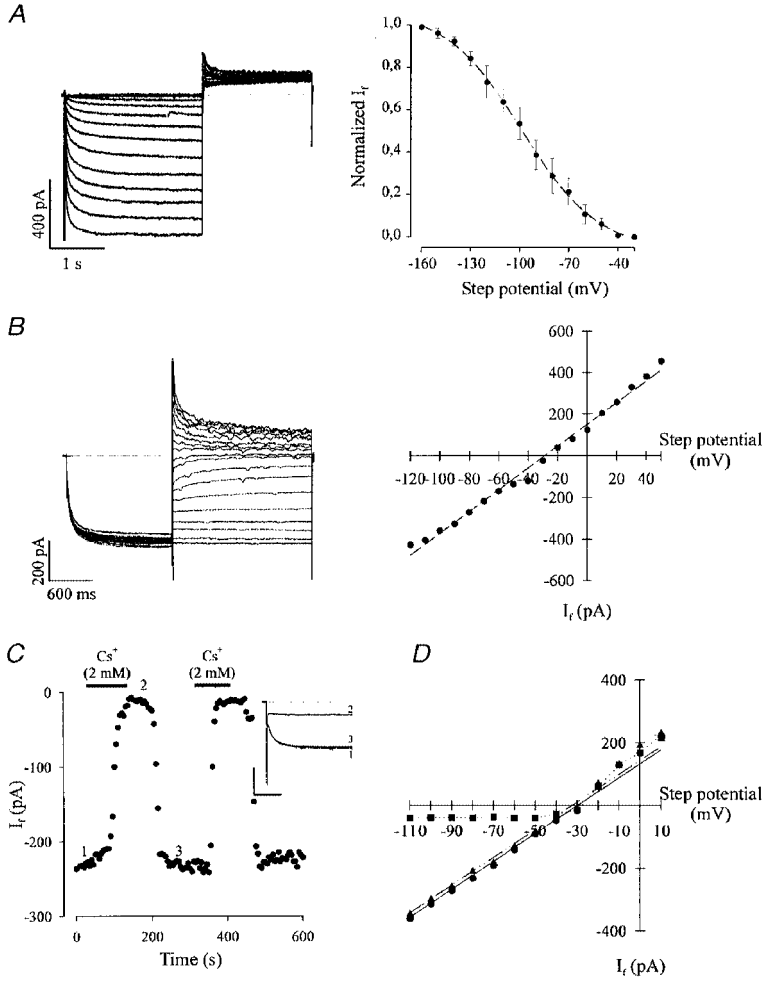

Figure 1. Biophysical and pharmacological characterization of I_f in ES cell-derived cardiomyocytes

A, left panel, IDS cardiomyocyte displaying the steady-state activation characteristics tested by 2 s lasting hyperpolarizing voltage steps from −40 to −160 mV in 10 mV intervals and subsequent 2 s depolarization to +15 mV (holding potential −35 mV, the dotted line indicates the zero current level). The typical slow activation kinetics for I_f was observed, the threshold potential proved close to −50 mV. The right panel shows the steady-state activation characteristics of 3 representative experiments. Data points were obtained by measuring the peak of the deactivating tail currents, normalized and fitted with a Boltzmann curve yielding a V_h of −98·2 ± 1·1 mV. *B*, a representative *I–V* relationship of an EDS cardiomyocyte obtained by applying 2 s lasting prepulses to −115 mV (holding potential −35 mV) and subsequent 2 s lasting voltage steps ranging from −120 to +50 mV in 10 mV intervals. The *I–V* relationship (right panel) was determined by measuring I_f 15 ms after applying the depolarizing voltage steps. The linear regression of the data point yielded a reversal potential of −29 mV. *C*, extracellular application of Cs^+ (2 mM) resulted in a complete block of I_f (holding potential −35 mV, step potential −110 mV) in an EDS cardiomyocyte; this could be reversed by washout (left panel). The numbers in the time course diagram indicate when the current traces displayed in the inset (calibration bars correspond to 300 ms and 200 pA, respectively) were recorded (the current in the presence of Cs^+ was taken as reference value for the estimation of I_f amplitude). The right panel shows the *I–V* relation of I_f in absence and presence of extracellular Cs^+ (2 mM) in an EDS cardiomyocyte. Cs^{2+} almost completely blocked I_f at potentials negative to the reversal potential (the linear regression yielded −30 mV prior to application of Cs^+ and −32·5 mV after washout of Cs^+) whereas positive to −30 mV no effect of Cs^+ on I_f was observed (●, control; ■, in the presence of 2 mM CsCl; ▲, after washout of CsCl).

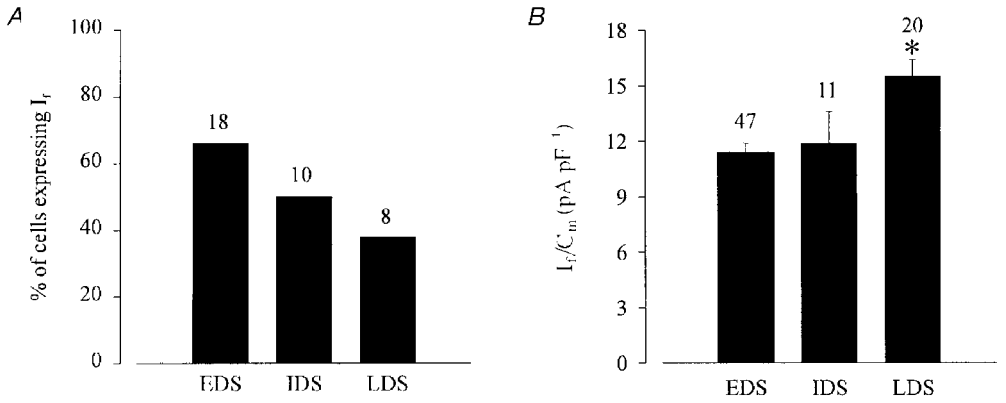

Figure 2. Functional expression of I_f in ES cell-derived cardiomyocytes at different stages of development

A, the percentage of cells expressing I_f declined during cardiomyogenesis. *B*, at later stages of development an increase in the current density (I_f/C_m, where C_m is membrane capacitance) of I_f was observed. I_f was evoked by applying a 2 s hyperpolarizing voltage step from a holding potential of −35 to −110 mV (* statistically significantly different to EDS and IDS at the $P < 0{\cdot}05$ level using Student's unpaired t test). Numbers above bars are numbers of replicates.

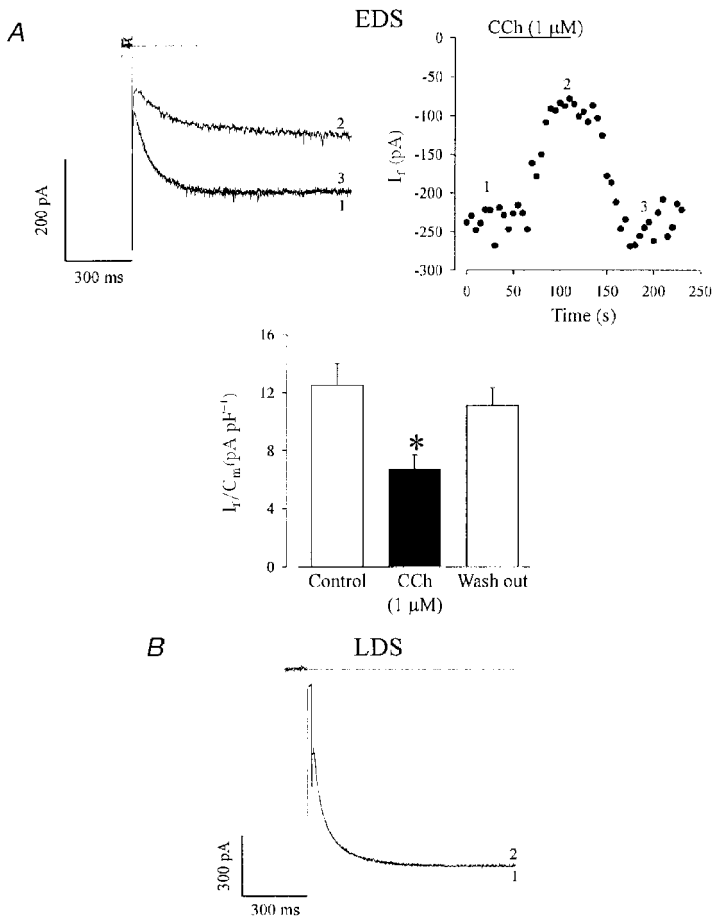

Figure 3. Carbachol (CCh) strongly depresses the amplitude of basal I_f in EDS cardiomyocytes

A, CCh depressed basal I_f in a representative EDS cardiomyocyte. The effect was completely reversed upon washout (left panel). This is also seen in the time course of this experiment (right panel), the numbers indicate the traces displayed in *A* (the instantaneous current in presence of CCh was taken as reference for the estimation of the I_f amplitude). The effect of CCh on I_f in EDS cells ($n = 5$) is summarized in the histogram. * Statistically significantly different to control at the $P < 0{\cdot}05$ level (paired t test). *B*, in LDS cardiomyocytes CCh did not depress basal I_f. (Voltage protocol identical to Fig. 2). 1, control; 2, CCh (1 μM).

I_f density upon application of ISO: $11{\cdot}2 \pm 2{\cdot}2$ pA pF^{-1}, $n = 5$). Figure 3*A* shows the current traces (left panel) and corresponding time course (right panel) of the effect of CCh (1 μM) on I_f in EDS cells. Similar to what was reported for $I_{Ca,L}$, CCh application resulted in a strong depression of I_f ($45{\cdot}7 \pm 6{\cdot}5\%$, $n = 5$) and this effect could be completely reversed upon washout of CCh. A summary (Fig. 3*A*, histogram) of I_f densities obtained under control condition ($12{\cdot}5 \pm 1{\cdot}5$ pA pF^{-1}), in the presence of CCh (1 μM) ($6{\cdot}7 \pm 1$ pA pF^{-1}) and upon washout ($11{\cdot}1 \pm 1{\cdot}2$ pA pF^{-1}) is shown.

In Fig. 4*B* current traces (left panel) and corresponding time course (right panel) of a typical experiment in an LDS cardiomyocyte are displayed, where I_f (recorded at -110 mV) was stimulated upon extracellular application of ISO (1 μM). I_f current densities in control condition and in the presence of ISO were $14{\cdot}2 \pm 3{\cdot}4$ pA pF^{-1} ($n = 6$) and $18{\cdot}2 \pm 3{\cdot}9$ pA pF^{-1} ($n = 6$), respectively (Fig. 4*B*, histogram); thus, the stimulation amounted to $33{\cdot}0 \pm 5{\cdot}2\%$. It should be noted that the stimulation of I_f by ISO in LDS cells was mimicked by extracellular application of forskolin (1 μM), a direct activator of adenylyl cyclase. Forskolin stimulated I_f by $31{\cdot}0 \pm 2{\cdot}0\%$ ($n = 4$, data not shown).

We conclude that CCh depresses basal I_f in EDS but not in LDS cells, whereas ISO stimulates I_f in LDS but not in EDS cells. Furthermore, these results clearly show that the regulation of I_f parallels that of $I_{Ca,L}$ during the course of cardiomyogenesis.

I_f is stimulated by phosphorylation via PKA in EDS cells

Since I_f was shown to be stimulated both by a direct interaction with cAMP, as reported for the sinus node (DiFrancesco, 1995*a*; Bois *et al.* 1997; DiFrancesco, 1999), and by cAMP-dependent phosphorylation via PKA, as found in Purkinje fibres (Chang *et al.* 1991; Yu *et al.* 1993) we examined the effect of the catalytic subunit of PKA (Fig. 5, left panels, C-subunit) as well as of cAMP analogues (Fig. 5, right panels) on I_f during early and late developmental stages of cardiomyogenesis. Figure 5*A* (left

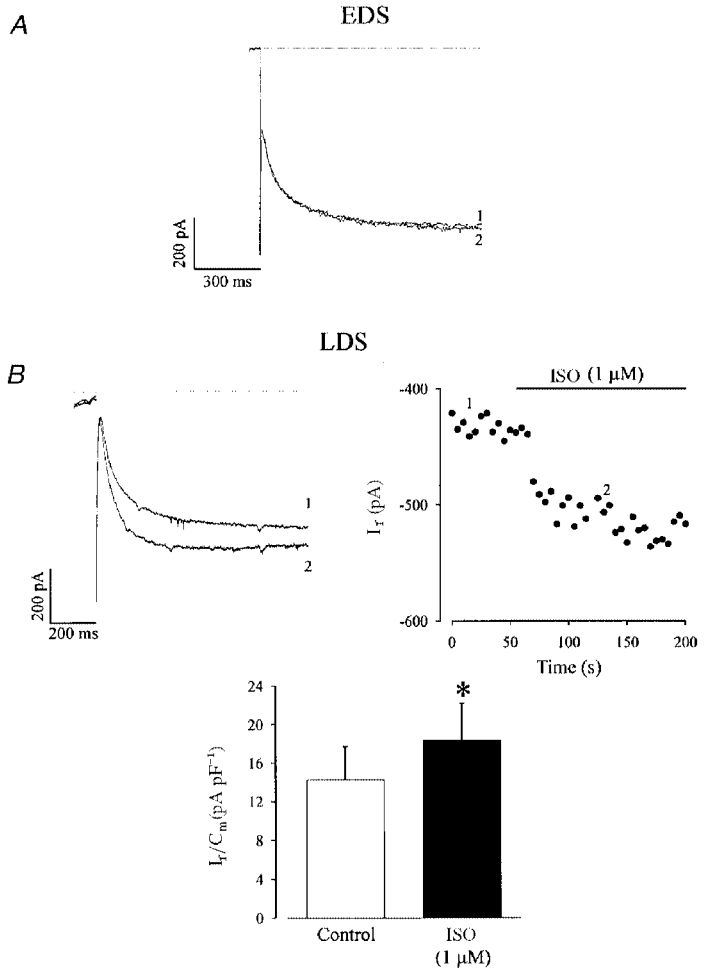

Figure 4. Isoprenaline (ISO) stimulates I_f in LDS cardiomyocytes

A, ISO (1 μM) did not stimulate I_f in EDS cardiomyocytes, 1, control; 2, ISO (1 μM). *B*, ISO stimulated I_f in LDS cardiomyocytes (left panel: original recordings; right panel: time course of this experiment, voltage protocol identical to Fig. 2). * Difference in current density between the absence and the presence of ISO was statistically significant (*B*, histogram, $n = 6$; $P < 0{\cdot}05$, paired t test).

panel) shows a representative experiment where intracellular application of the catalytic subunit of PKA (7 μM) increased the amplitude of I_f in an EDS cardiomyocyte. The time course of this experiment displays a pronounced stimulation of the amplitude of I_f with a maximal effect obtained around 3 min after establishment of the whole-cell configuration (Fig. 5*B*, left panel), as expected for the diffusion kinetics of PKA through the opening of the patch pipette. On average, PKA stimulated I_f by $37{\cdot}0 \pm 2{\cdot}9\%$ ($n = 4$) in all EDS cells tested. I_f densities under control conditions (immediately after break in) and after 5 min of cell dialysis with the C-subunit of PKA were $10{\cdot}9 \pm 1{\cdot}6$ and $14{\cdot}8 \pm 2{\cdot}0$ pA pF^{-1} ($n = 4$), respectively (Fig. 5*C*, left panel). Similarly, a stimulation of I_f amplitude was observed upon extracellular application of 8 bromo-cAMP (200 μM), a membrane-permeable analogue of cAMP (Fig. 5*A* and *B*,

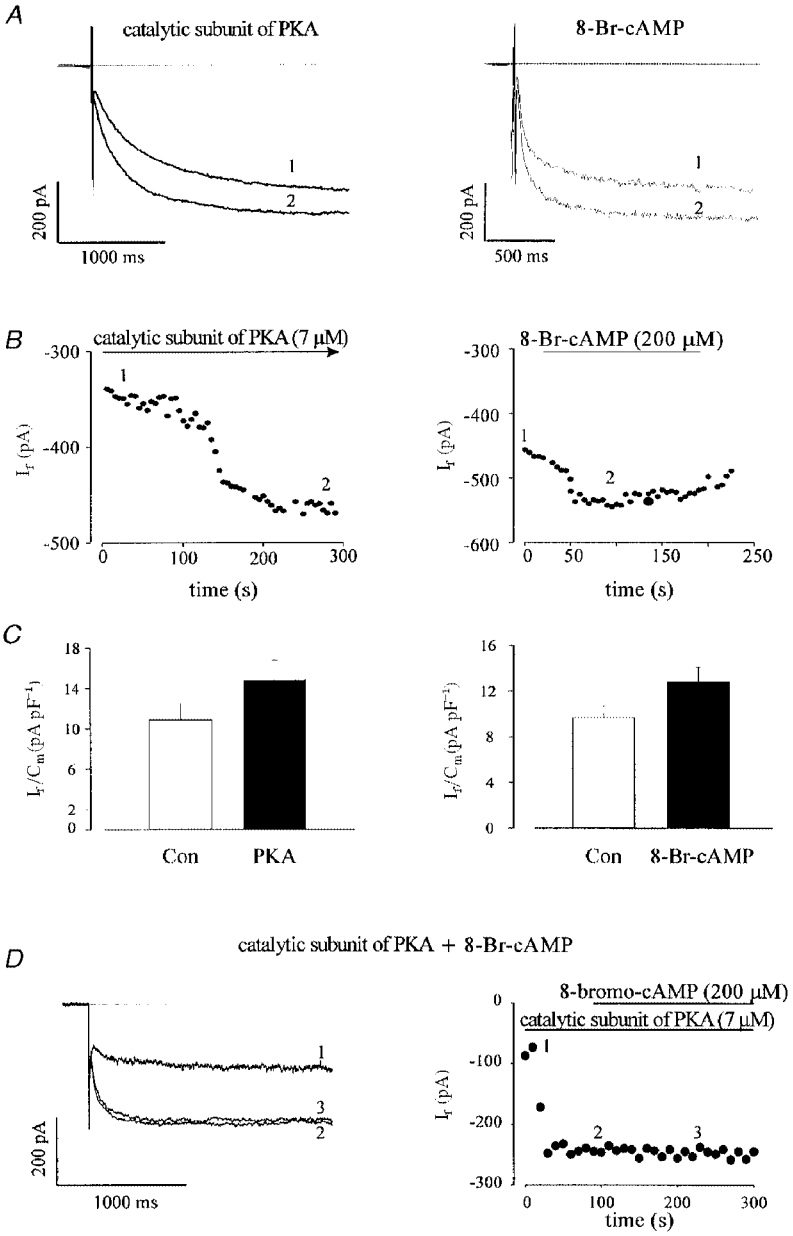

Figure 5. The catalytic subunit of PKA (C-subunit of PKA, 7 μM) and 8-bromo-cAMP (200 μM) stimulate I_f in EDS cardiomyocytes

A, the C-subunit of PKA (7 μM through pipette, left panel) as well as 8-bromo-cAMP (8-Br-cAMP 200 μM, extracellular application, right panel) stimulated I_f in EDS cells. The corresponding time courses are shown in *B*. The effects of C-subunit of PKA ($n = 4$) and 8-Br-cAMP ($n = 5$) on I_f are summarized in the left and right panels of *C*, respectively. * Statistically significantly different to control (Con) at the $P < 0{\cdot}05$ level (paired t test). *D* shows that 8-Br-cAMP has no additional effect on I_f after activation of the current by the C-subunit of PKA (7 μM): left panel, current traces (1 control, 2 C-subunit of PKA, 3 C-subunit of PKA + 8-bromo-cAMP); right panel, corresponding time course.

right panels). I_f densities under control conditions and after extracellular application of 8 Br-cAMP were $9{\cdot}7 \pm 1{\cdot}0$ ($n = 5$) and $12{\cdot}8 \pm 1{\cdot}4$ pA pF^{-1} ($n = 5$), respectively (Fig. 5*C*, right panel). In order to evaluate PKA-mediated stimulation of I_f *vs.* direct cAMP binding further, two sets of additional experiments were performed. First, we tested whether I_f could be additionally increased by 8-bromo-cAMP (200 μM) in the presence of the catalytic subunit of PKA (7 μM) in the pipette solution. As can be seen in Fig. 5*D* 8-bromo-cAMP did not have any additional stimulatory effect on I_f under such conditions ($n = 4$), demonstrating that, if present, a direct cAMP stimulation of I_f is not additive with a maximal indirect stimulation of the current via PKA. Second, the effect of 8-bromo-cAMP was examined in the presence of PKI, a highly selective peptide inhibitor of PKA (Cheng *et al.* 1986; Skeberdis *et al.* 1997). Under intracellular perfusion with PKI (20 μM, intracellular solution) in EDS cells, a strong depression of I_f was observed demonstrating a high constitutive PKA activity in these cells. However, 8-bromo-cAMP had no effect on I_f under these conditions ($n = 2$, data not shown). These results demonstrate that I_f is stimulated by phosphorylation via the cAMP-dependent PKA in EDS cells.

β-adrenergic regulation of I_f is due to phosphorylation via PKA in LDS cells

The effect of β-adrenergic agonists on $I_{Ca,L}$ is related to an increase in adenylyl cyclase activity, a rise in cAMP levels and phosphorylation via PKA in LDS cells (Ji *et al.* 1999; Maltsev *et al.* 1999). Since in LDS cells β-adrenoceptors

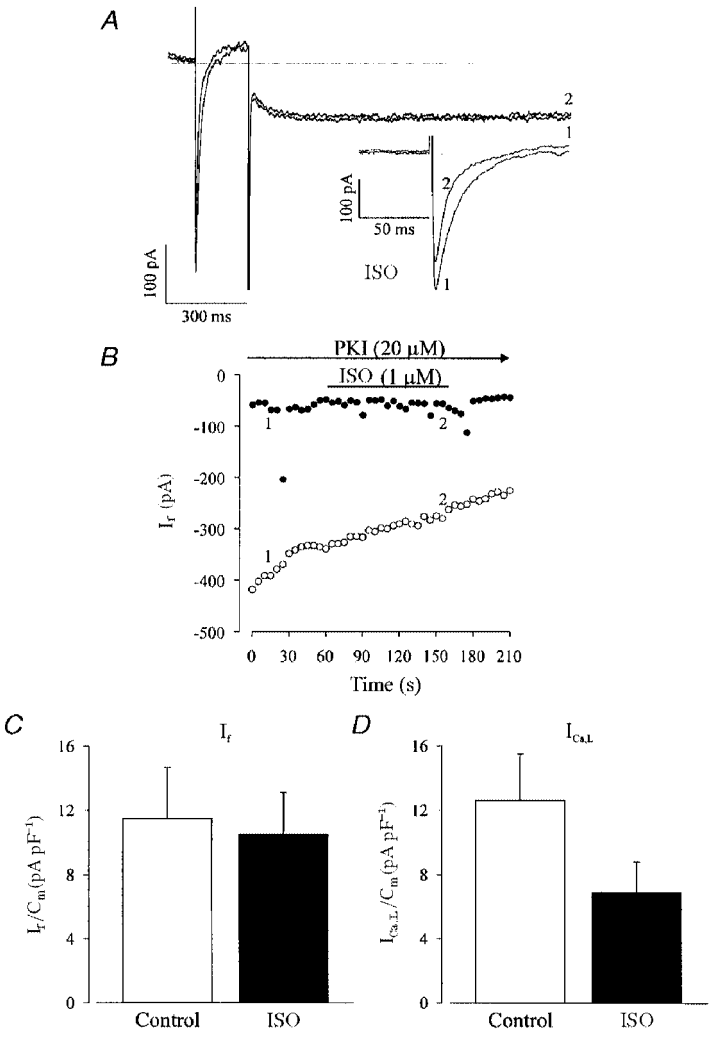

Figure 6. Effect of a selective peptide inhibitor of cAMP-dependent protein kinase A (PKI, 20 μM) on the ISO-stimulated I_f and L-type Ca^{2+} current ($I_{Ca,L}$) in LDS cells

A, ISO neither stimulated I_f nor $I_{Ca,L}$ in the presence of PKI (1 PKI, 2 PKI and ISO). The inset in *A* displays $I_{Ca,L}$ at a more extended time scale. Simultaneous recording of $I_{Ca,L}$ and I_f consisted of a depolarizing step from a holding potential of −50 to 0 mV for 200 ms and a subsequent hyperpolarization to −110 mV for 2 s. *B*, time course of the experiment displayed in *A*. The I_f (●) amplitude remained unaltered upon addition of ISO whereas the pronounced decrease in $I_{Ca,L}$ (○) was due to run down. A statistical summary of these data demonstrates that neither I_f (*C*; $n = 6$) nor $I_{Ca,L}$ (*D*; $n = 4$) was stimulated by ISO in the presence of PKI.

were functionally coupled resulting in the stimulation of both I_f and $I_{Ca,L}$ by ISO (1 μM), we next examined whether the effect on I_f was mediated through a direct cAMP binding and/or an indirect phosphorylation via cAMP-dependent PKA at this developmental stage. In order to differentiate between these two modulatory pathways, we tested again the effect of an intracellular perfusion with PKI (Fig. 6). The simultaneous recording of $I_{Ca,L}$ and I_f allowed us to test the efficacy of PKI in blocking the stimulation of $I_{Ca,L}$ by ISO. The current traces of $I_{Ca,L}$ and I_f (Fig. 6*A*) as well as the time course of this experiment (Fig. 6*B*) demonstrate that intracellular infusion with PKI (20 μM) prevented the stimulatory effects of ISO (1 μM) on both I_f and $I_{Ca,L}$. A summary of these data are shown in Fig. 6*C*, where I_f densities under control conditions and after dialysis with PKI and in presence of ISO (1 μM) amounted to $11{\cdot}5 \pm 3{\cdot}2$ pA pF^{-1} ($n = 6$) and $10{\cdot}5 \pm 2{\cdot}6$ pA pF^{-1} ($n = 6$), respectively. For $I_{Ca,L}$, the current densities were $12{\cdot}6 \pm 2{\cdot}7$ pA pF^{-1} for control condition (after dialysis with PKI) and $6{\cdot}9 \pm 1{\cdot}9$ pA pF^{-1} ($n = 4$) after superfusion of the cell with ISO (1 μM) (Fig. 6*D*). The decrease of $I_{Ca,L}$ density is due to current run down.

Altogether, these experiments demonstrate that during early and late stages of cardiomyogenesis I_f is stimulated by phosphorylation via cAMP-dependent protein kinase.

Effects of PDE inhibitors on I_f in EDS cells

In our previous study investigating the regulation of $I_{Ca,L}$ by cAMP-dependent pathways we showed that the high intrinsic basal adenylyl cyclase activity in EDS cells is

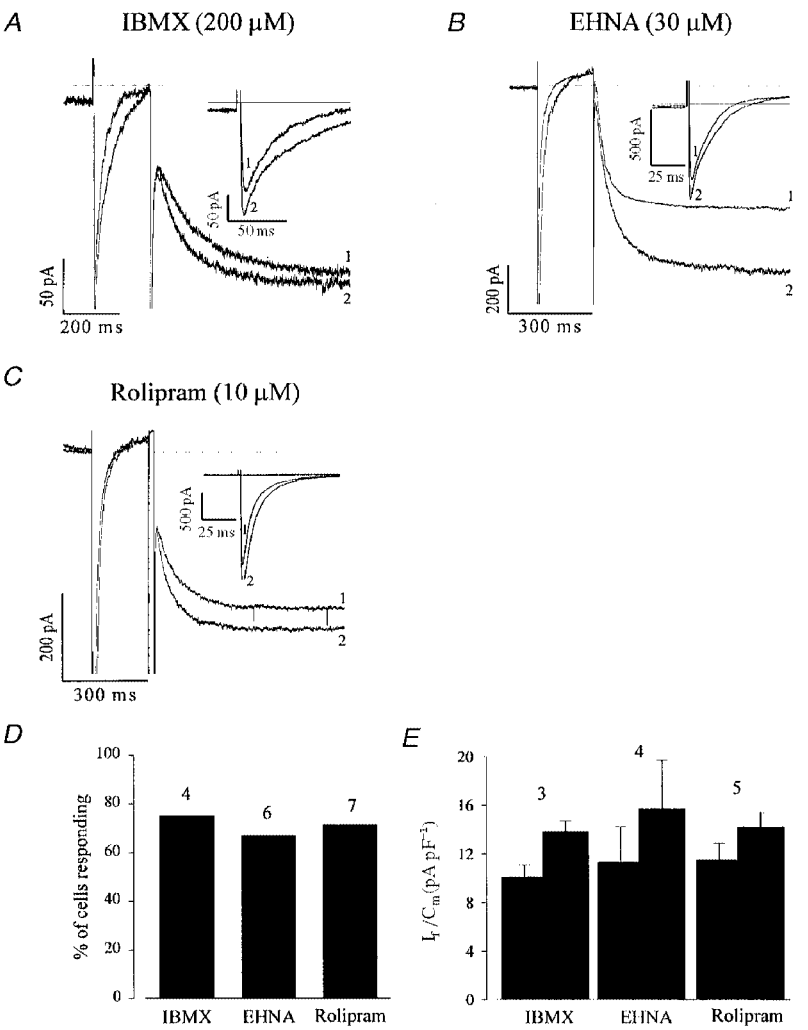

Figure 7. The non-selective phosphodiesterase (PDE) inhibitor IBMX as well as PDE2 and PDE4 antagonists stimulate I_f and $I_{Ca,L}$ in EDS cardiomyocytes

A–C, the non-selective antagonist IBMX (200 μM; *A*), the PDE2 selective antagonist EHNA (30 μM; *B*) and the PDE4 antagonist rolipram (10 μM; *C*) stimulate both I_f and $I_{Ca,L}$ in EDS cardiomyocytes. The same voltage protocol as in Fig. 6 was used. The insets display $I_{Ca,L}$ at a more extended time scale. Due to the slight delay in the maximal response of I_f as compared with $I_{Ca,L}$ and the fast run down of the latter, the $I_{Ca,L}$ traces displayed in *B* and *C* were recorded prior to I_f. *D* shows that most of the EDS cardiomyocytes tested responded to PDE antagonist application with a stimulation of I_f and $I_{Ca,L}$. *E* shows that an increase in I_f density was also observed under these conditions.

counterbalanced by a high intrinsic PDE activity due to PDE2–4 subtypes (Maltsev *et al.* 1999). Thus in the present study, we have investigated which particular PDE subtype regulates I_f and whether there are any differences in the regulation of $I_{Ca,L}$ and I_f by PDEs. Simultaneous recordings of $I_{Ca,L}$ and I_f were performed. Figure 7*A* illustrates the effects of extracellular application of IBMX (200 μM), a non-selective PDE inhibitor on I_f and $I_{Ca,L}$ in EDS cells. As can be seen, IBMX stimulates $I_{Ca,L}$ as well as I_f (the inset shows on a more extended time scale the stimulation of $I_{Ca,L}$ by IBMX). On average, IBMX (200 μM) stimulated I_f in three out of four cells tested (Fig. 7*D*) by $39{\cdot}3 \pm 18{\cdot}1$% and $I_{Ca,L}$ by $48{\cdot}8 \pm 13{\cdot}8$% ($n = 3$). The I_f densities in control conditions and in presence of IBMX amounted to $10{\cdot}1 \pm 1{\cdot}0$ and $13{\cdot}8 \pm 0{\cdot}9$ pA pF^{-1} ($n = 3$), respectively (Fig. 7*E*). This clearly demonstrates that I_f, like $I_{Ca,L}$, is controlled by an intrinsic PDE activity in EDS cells. In order to determine which type(s) of PDEs regulate(s) I_f in EDS cardiomyocytes, we examined the effects of specific inhibitors of the PDE2–4 isoforms. Figure 7*B* and *C* show that EHNA (30 μM), a selective PDE2 antagonist (Méry *et al.* 1995), and rolipram (10 μM), a selective PDE4 antagonist (Fischmeister & Hartzell, 1991), increased the $I_{Ca,L}$ as well as the I_f amplitude in EDS cells. On average, EHNA (30 μM) and rolipram (10 μM) stimulated I_f by $45{\cdot}7 \pm 17{\cdot}0$ ($n = 4$) and $25{\cdot}4 \pm 4{\cdot}6$% ($n = 5$), respectively (Fig. 7*D*), whereas they stimulated $I_{Ca,L}$ by $45{\cdot}4 \pm 22{\cdot}0$ and $54{\cdot}0 \pm 29{\cdot}0$%, respectively. I_f densities in control conditions and in the presence of EHNA

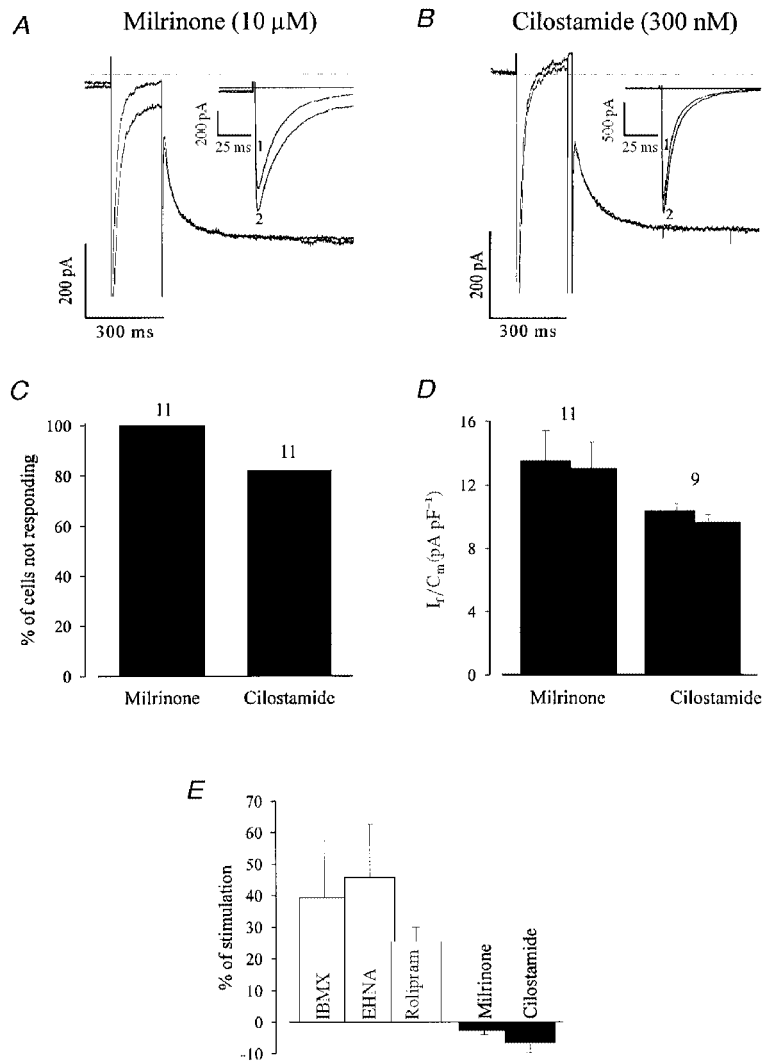

Figure 8. The PDE3 antagonists milrinone and cilostamide stimulate $I_{Ca,L}$ but not I_f in EDS cardiomyocytes

A and *B*, milrinone and cilostamide stimulated $I_{Ca,L}$ (see also inset for a more extended time scale), but I_f remains unaltered (voltage protocol identical to Fig. 6). *C*, in the large majority of cells I_f is not stimulated upon PDE3 antagonist application. *D*, the current density of the non-responding cells decreased slightly in the presence of PDE inhibitors indicating a small run down. *E*, a quantitative comparison of the percentage of I_f density stimulation demonstrates that I_f was stimulated by PDE2 and 4 antagonists, but not by PDE3 antagonists.

were $11{\cdot}3 \pm 2{\cdot}9$ and $15{\cdot}7 \pm 4{\cdot}0$ pA pF^{-1} ($n = 4$) and for control *vs.* rolipram they were $11{\cdot}5 \pm 1{\cdot}4$ and $14{\cdot}2 \pm 1{\cdot}2$ pA pF^{-1} ($n = 5$), respectively (Fig. 7*E*).

Unlike IBMX and PDE2 and/or PDE4 inhibitors, the PDE3 selective antagonists milrinone (10 μM, Fig. 8*A*) and cilostamide (300 nM, Fig. 8*B*) stimulated $I_{Ca,L}$ but not I_f in the EDS cells. Milrinone (100%, $n = 11$) as well as cilostamide (82%, $n = 11$) had no stimulatory effect on I_f (Fig. 8*C*), whereas they stimulated $I_{Ca,L}$ by $36{\cdot}0 \pm 5{\cdot}4$ and $39{\cdot}0 \pm 5{\cdot}8$%, respectively. In the case of cilostamide, a small ($9{\cdot}9 \pm 3{\cdot}9$%) stimulation of I_f was observed in 2 out of 11 cells (Fig. 8*D*). These data confirm that under basal conditions, PDE2 and PDE4, but not PDE3 regulate I_f, whereas all three PDE subtypes regulate $I_{Ca,L}$.

DISCUSSION

Until now little has been known about the biophysical and pharmacological properties of I_f during heart development. I_f was shown to be expressed in 3-day-old embryonic chick ventricular myocytes with a current density decreasing at day 10 and becoming almost undetectable at day 17 (Satoh & Sperelakis, 1991, 1993). Furthermore, I_f was found to be stimulated by ISO and depressed by CCh in young embryonic chick ventricular myocytes (Satoh & Sperelakis, 1993). A recent report (Liu *et al.* 1996) showed that most of the characteristics of I_f in cultured sino-atrial node cells were similar to those measured in freshly isolated pacemaker cells. Here, we report, that the percentage of spontaneously beating cells expressing I_f during early stages of cardiomyogenesis is high, indicating that this ion channel may play an important role at this developmental stage. With further cellular specialization, atrial as well as ventricular cardiomyocytes obviously downregulate I_f, whereas in cells of the conduction system current density increases. The fact that in ventricular cardiomyocytes of patients with failing hearts an increased current density of I_f is detected (Cerbai *et al.* 1997; Hoppe & Beuckelmann, 1998) suggests that these cells may dedifferentiate and recover their embryonic phenotype.

I_f has been proposed to play an important role in the regulation of the heart rhythmicity modulated by the autonomous nervous system (DiFrancesco, 1995*a*). While there is general agreement that the modulation is mediated by adenylyl cyclase activity, it is still a matter of debate whether direct nucleotide (cAMP) binding or phosphorylation by PKA is the main mechanism involved. Phosphorylation of f-channels via cAMP-dependent PKA has been reported in Purkinje fibres (Chang *et al.* 1991; Yu *et al.* 1995), whereas a direct action of cAMP on f-channels has been proposed in the sinus node (DiFrancesco & Tortora, 1991; DiFrancesco, 1995*b*; Fares *et al.* 1998) as well as for heterologously expressed f-channels (Ludwig *et al.* 1999). We have therefore examined the regulation of I_f during various stages of cardiomyogenesis. Since in common with the early murine heart (E11.5) (An *et al.* 1996) the β-adrenoceptor response is absent at the EDS cell stage, we investigated in these cardiomyocytes the action of the catalytic subunit of PKA on I_f. Our data clearly demonstrate a stimulation of I_f by PKA suggesting that at this stage phosphorylation via cAMP-dependent PKA is involved. Interestingly, our negative results obtained with 8-bromo-cAMP, used either after a maximal PKA activation or after a complete block of PKA by PKI, a selective peptide inhibitor of PKA, does not provide any evidence for a direct regulation of I_f by cAMP binding to the f-channel in EDS cells. Likewise, at the LDS stage, the reproducible stimulation of $I_{Ca,L}$ and I_f upon β-adrenoceptor stimulation was abolished upon intracellular perfusion with PKI. Thus, during early and late stages of cardiomyogenesis the activation of f-channels appears to be mediated by phosphorylation via PKA rather than by direct cAMP binding. However, a possibility remains that phosphorylation of the channels by PKA is a prerequisite for a direct activation of the channel by cAMP at these developmental stages. In support of this hypothesis is our finding that muscarinic depression (in EDS cells) and β-adrenergic stimulation (in LDS cells) of I_f were also accompanied by a slowing and acceleration of the activation kinetics, respectively. This may indicate the presence of a small component of direct cAMP modulation acting in concert with a more robust PKA modulation. Furthermore, minor effects of PKI and/or the catalytic subunit of PKA onto cAMP levels, though unlikely, cannot be ruled out completely. Thus, in order to quantitatively assess the possible direct cAMP- *versus* the PKA-mediated effect in embryonic cardiomyocytes, future experiments should address whether I_f is modified by shifting the activation curve on the voltage axis or by a change in conductance. The former is observed in the case of direct cAMP binding. It remains to be examined further in future studies whether and when during postnatal development the switch towards an exclusively direct cAMP-mediated stimulation of I_f occurs and whether this is related to different types of f-channels or the expression of different splice variants. Our previous studies on the regulation of $I_{Ca,L}$ during cardiac embryogenesis demonstrated a high intrinsic activity for the PDE2–4 subtypes at the EDS cell stage (Maltsev *et al.* 1999). This is most likely correlated to the high intrinsic adenylyl cyclase activity. We therefore examined in this study the role of PDE2–4 subtypes in controlling I_f by using selective PDE antagonists. The simultaneous recording of $I_{Ca,L}$ and I_f allowed us to examine whether any difference exists in the regulation of these two ion channels. IBMX, a non-selective PDE inhibitor, as well as PDE2 and PDE4 antagonists stimulated both $I_{Ca,L}$ and I_f, confirming the role of basal PDE activity in EDS cells. These findings are in line with a previous report in Purkinje fibres (Chang *et al.* 1991), where IBMX was found to increase the amplitude of I_f. However, in contrast to $I_{Ca,L}$, I_f was not stimulated by selective PDE3 inhibitors. This differential regulation of $I_{Ca,L}$ and I_f may indicate that the relative contribution of PDE3 to the total PDE activity is reduced near f-channels as compared with Ca^{2+} channels,

either due to a reduced PDE3 activity or due to an increased PDE2 and/or PDE4 activity. More recently, in rat ventricular cells, we found that neither a PDE3 nor a PDE4 inhibitor when used alone had any effect on basal $I_{Ca,L}$, while when both inhibitors were used together a strong stimulation of $I_{Ca,L}$ was observed (Verde *et al.* 1999). Additional data from this study on basal and stimulated $I_{Ca,L}$ led us to conclude that each PDE played a specific role in regulating $I_{Ca,L}$, with the rank order of potency being PDE4 > PDE3 > PDE2 > PDE1. Our present results on I_f regulation in EDS cells would tend to suggest that PDE subtypes regulate I_f with a rank order of potency of PDE2 > PDE4 > PDE3. Our previous study in EDS cells would lead to a different order of potency for the regulation of $I_{Ca,L}$: PDE3 > PDE2 = PDE4. This different qualitative and/or quantitative arrangement of PDE isoforms near the two types of channels may lead to different local concentrations of cAMP. A precedent for this type of cAMP compartmentation exists in the case of β-adrenergic regulation of Ca^{2+} channels in the frog heart. In this preparation cAMP compartmentation is responsible for a local activation of Ca^{2+} channels by ISO (Jurevicius & Fischmeister, 1996). More recently, a preferential coupling of β2-adrenergic receptors with Ca^{2+} channels rather than with K^+ channels was found in adult transgenic mouse ventricular myocytes (An *et al.* 1999). Thus, compartmentation and/or colocalization of ion channels and signalling molecules may provide a general mechanism allowing the specific regulation of one type of ion channel without altering the activity of others.

An, R. H., Davies, M. P., Doevendans, P. A., Kubalak, S. W., Bangalore, R., Chien, K. R. & Kass, R. S. (1996). Developmental changes in beta-adrenergic modulation of L-type Ca^{2+} channels in embryonic mouse heart. *Circulation Research* **78**, 371–378.

An, R. H., Heath, B. M., Koch, W., Lefkowitz, R. J. & Kass, R. S. (1999). β_2-Adrenergic receptor overexpression in the developing mouse heart: evidence for targeted modulation of ion channels. *Journal of Physiology* **516**, 19–30.

Bois, P. & Lenfant, J. (1990). Isolated cells of the frog sinus venosus: properties of the inward current activated during hyperpolarization. *Pflügers Archiv* **416**, 339–346.

Bois, P., Renaudon, B., Baruscotti, M., Lenfant, J. & DiFrancesco, D. (1997). Activation of f-channels by cAMP analogues in macropatches from rabbit sino-atrial node myocytes. *Journal of Physiology* **501**, 565–571.

Callewaert, G., Carmeliet, E. & Vereecke, J. (1984). Single cardiac Purkinje cells: general electrophysiology and voltage-clamp analysis of the pace-maker current. *Journal of Physiology* **349**, 643–661.

Cerbai, E., Barbieri, M. & Mugelli, A. (1994). Characterization of the hyperpolarization-activated current, I_f, in ventricular myocytes isolated from hypertensive rats. *Journal of Physiology* **481**, 585–591.

Cerbai, E., Barbieri, M. & Mugelli, A. (1996). Occurrence and properties of the hyperpolarization-activated current I_f in ventricular myocytes from normotensive and hypertensive rats during aging. *Circulation* **94**, 1674–1681.

Cerbai, E., Pino, R., Porciatti, F., Sani, G., Toscano, M., Maccherini, M., Giunti, G. & Mugelli, A. (1997). Characterization of the hyperpolarization-activated current, I(f), in ventricular myocytes from human failing heart. *Circulation* **95**, 568–571.

Chang, F., Cohen, I. S., DiFrancesco, D., Rosen, M. R. & Tromba, C. (1991). Effects of protein kinase inhibitors on canine Purkinje fibre pacemaker depolarization and the pacemaker current i_f. *Journal of Physiology* **440**, 367–384.

Cheng, H. C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., van Patten, S. M. & Walsh, D. A. (1986). A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. *Journal of Biological Chemistry* **261**, 989–992.

Davies, M. P., An, R. H., Doevendans, P., Kubalak, S., Chien, K. R. & Kass, R. S. (1996). Developmental changes in ionic channel activity in the embryonic murine heart. *Circulation Research* **78**, 15–25.

DiFrancesco, D. (1986). Characterization of single pacemaker channels in cardiac sino-atrial node cells. *Nature* **324**, 470–473.

DiFrancesco, D. (1993). Pacemaker mechanisms in cardiac tissue. *Annual Review of Physiology* **55**, 455–472.

Difrancesco, D. (1995*a*). The onset and autonomic regulation of cardiac pacemaker activity: relevance of the f current. *Cardiovascular Research* **29**, 449–456.

Difrancesco, D. (1995*b*). The pacemaker current (I(f)) plays an important role in regulating SA node pacemaker activity. *Cardiovascular Research* **30**, 307–308.

DiFrancesco, D. (1999). Dual allosteric modulation of pacemaker (f) channels by cAMP and voltage in rabbit SA node. *Journal of Physiology* **515**, 367–376.

DiFrancesco, D. & Ferroni, A. (1983). Delayed activation of the cardiac pacemaker current and its dependence on conditioning pre-hyperpolarizations. *Pflügers Archiv* **396**, 265–267.

DiFrancesco, D., Noma, A. & Trautwein, W. (1980). Separation of current induced by potassium accumulation from acetylcholine-induced relaxation current in the rabbit S-A node. *Pflügers Archiv* **387**, 83–90.

DiFrancesco, D. & Tortora, P. (1991). Direct activation of cardiac pacemaker channels by intracellular cyclic AMP. *Nature* **351**, 145–147.

DiFrancesco, D. & Tromba, C. (1987). Acetylcholine inhibits activation of the cardiac hyperpolarizing-activated current, i_f. *Pflügers Archiv* **410**, 139–142.

DiFrancesco, D. & Tromba, C. (1988). Muscarinic control of the hyperpolarization-activated current (i_f) in rabbit sino-atrial node myocytes. *Journal of Physiology* **405**, 493–510.

Fares, N., Bois, P., Lenfant, J. & Potreau, D. (1998). Characterization of a hyperpolarization-activated current in dedifferentiated adult rat ventricular cells in primary culture. *Journal of Physiology* **506**, 73–82.

Fischmeister, R. & Hartzell, H. C. (1991). Cyclic AMP phosphodiesterases and Ca^{2+} current regulation in cardiac cells. *Life Science* **48**, 2365–2376.

Gauss, R., Seifert, R. & Kaupp, U. B. (1998). Molecular identification of a hyperpolarization-activated channel in sea urchin sperm. *Nature* **393**, 583–587.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

Hescheler, J., Fleischmann, B. K., Lentini, S., Maltsev, V. A., Rohwedel, J., Wobus, A. M. & Addicks, K. (1997). Embryonic stem cells: a model to study structural and functional properties in cardiomyogenesis. *Cardiovascular Research* **36**, 149–162.

Hoppe, U. C. & Beuckelmann, D. J. (1998). Characterization of the hyperpolarization-activated inward current in isolated human atrial myocytes. *Cardiovascular Research* **38**, 788–801.

Isenberg, G. & Klöckner, U. (1982). Calcium tolerant ventricular myocytes prepared by preincubation in a 'KB medium'. *Pflügers Archiv* **395**, 6–18.

Ji, G. J., Fleischmann, B. K., Bloch, W., Feelisch, M., Andressen, C., Addicks, K. & Hescheler, J. (1999). Regulation of the L-type Ca^{2+}-channel during cardiomyogenesis: Switch from NO to adenylyl-cyclase-mediated inhibition. *FASEB Journal* **13**, 313–324.

Jurevicius, J. & Fischmeister, R. (1996). cAMP compartmentation is responsible for a local activation of cardiac Ca^{2+} channels by beta-adrenergic agonists. *Proceedings of the National Academy of Sciences of the USA* **93**, 295–299.

Lindemann, J. P. & Watanabe, A. M. (1990). Sympathetic control of cardiac electrical activity. In *Cardiac Electrophysiology: From Cell to Bedside*, ed. Zipes, D. P. & Jalife, J., pp. 277–283. Saunders, Philadelphia, PA, USA.

Liu, Z. W., Zou, A. R., Demir, S. S., Clark, J. W. & Nathan, R. D. (1996). Characterization of a hyperpolarization-activated inward current in cultured pacemaker cells from the sinoatrial node. *Journal of Molecular and Cellular Cardiology* **28**, 2523–2535.

Ludwig, A., Zong, X., Jeglitsch, M., Hofmann, F. & Biel, M. (1998). A family of hyperpolarization-activated mammalian cation channels. *Nature* **393**, 587–591.

Ludwig, A., Zong, X., Stieber, J., Hullin, R., Hofmann, F. & Biel, M. (1999). Two pacemaker channels from human heart with profoundly different activation kinetics. *EMBO Journal* **18**, 2323–2329.

Maltsev, V. A., Ji, G. J., Wobus, A. M., Fleischmann, B. K. & Hescheler, J. (1999). Establishment of β-adrenergic modulation of L-type calcium current in the early stages of cardiomyocyte development. *Circulation Research* **84**, 136–145.

Maltsev, V. A., Wobus, A. M., Rohwedel, J., Bader, M. & Hescheler, J. (1994). Cardiomyocytes differentiated *in vitro* from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. *Circulation Research* **75**, 233–244.

Méry, P.-F., Pavoine, C., Pecker, F. & Fischmeister, R. (1995). Erythro-9-(2-hydroxy-3-nonyl)adenine inhibits cyclic GMP-stimulated phosphodiesterase in isolated cardiac myocytes. *Molecular Pharmacology* **48**, 121–130.

Pappano, A. (1990). Parasympathetic control of cardiac electrical activity. In *Cardiac Electrophysiology: From Cell to Bedside*, ed. Zipes, D. P. & Jalife, J. J., pp. 271–276. Saunders, Philadelphia, PA, USA.

Renaudon, B., Bois, P., Bescond, J. & Lenfant, J. (1997). Acetylcholine modulates $I_{(f)}$ and $I_{K(ACh)}$ via different pathways in rabbit sino-atrial node cells. *Journal of Molecular and Cellular Cardiology* **29**, 969–975.

Robinson, R. B., Yu, H., Chang, F. & Cohen, I. S. (1997). Developmental change in the voltage-dependence of the pacemaker current, i_f, in rat ventricle cells. *Pflügers Archiv* **433**, 533–535.

Santoro, B., Liu, D. T., Yao, H., Bartsch, D., Kandel, E. R., Siegelbaum, S. A. & Tibbs, G. R. (1998). Identification of a gene encoding a hyperpolarization-activated pacemaker channel of brain. *Cell* **93**, 717–729.

Satoh, H. & Sperelakis, N. (1991). Identification of the hyperpolarization-activated inward current in young embryonic chick heart myocytes. *Journal of Developmental Physiology* **15**, 247–252.

Satoh, H. & Sperelakis, N. (1993). Hyperpolarization-activated inward current in embryonic chick cardiac myocytes: developmental changes and modulation by isoproterenol and carbachol. *European Journal of Pharmacology* **240**, 283–290.

Skeberdis, V. A., Jurevicius, J. & Fischmeister (1997). β_2-Adrenergic activation of L-type Ca^{2+} current in cardiac myocytes. *Journal of Pharmacological and Experimental Therapeutics* **283**, 452–461.

Verde, I., Vandecasteele, G., Lezoualc'h, F. & Fischmeister, R. (1999). Characterization of the cyclic nucleotide phosphodiesterase subtypes involved in the regulation of the L-type Ca^{2+} current in rat ventricular myocytes. *British Journal of Pharmacology* **127**, 65–74.

Vikstrom, K. L. & Leinwand, L. A. (1996). Contractile protein mutations and heart disease. *Current Opinion in Cell Biology* **8**, 97–105.

Wobus, A. M., Wallukat, G. & Hescheler, J. (1991). Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. *Differentiation* **48**, 173–182.

Yanagihara, K. & Irisawa, H. (1980). Inward current activated during hyperpolarization in the rabbit sinoatrial node cell. *Pflügers Archiv* **385**, 11–19.

Yu, H., Chang, F. & Cohen, I. S. (1993). Pacemaker current exists in ventricular myocytes. *Circulation Research* **72**, 232–236.

Yu, H., Chang, F. & Cohen, I. S. (1995). Pacemaker current i_f in adult canine cardiac ventricular myocytes. *Journal of Physiology* **485**, 469–483.

Zhou, Z. & Lipsius, S. L. (1992*a*). Properties of the pacemaker current (I_f) in latent pacemaker cells isolated from cat right atrium. *Journal of Physiology* **453**, 503–523.

Acknowledgements

We thank Dr A. M. Wobus for providing ES cells of the cell line D3, M. Bickel, C. Boettinger and B. Hops for assistance in cell culture work. The support of the machine and electronic shop is greatly acknowledged. This work was supported by a Procope France–Germany exchange programme.

The authors N. Abi-Gerges and G. J. Ji contributed equally to this manuscript.

Corresponding author

B. K. Fleischmann: Institute of Neurophysiology, University of Cologne, Robert-Koch-Strasse 39, D-50931 Cologne, Germany.

Email: akp17@uni-koeln.de