Cloning, expression, pharmacology and regulation of a delayed rectifier K⁺ channel in mouse heart

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Neonatal mouse cardiac poly(A)⁺ mRNA microinjection into *Xenopus* oocytes directed the expression of a delayed rectifier K⁺ current. A cDNA encoding this channel, called mIsK, was cloned from a neonatal mouse heart cDNA library whose properties were studied after expression of the complementary RNA in *Xenopus* oocytes. Among the different known K⁺ channel blockers, only the class III antiarrhythmic clofilium inhibited mIsK in the 10–100 µM range. The channel was completely insensitive to other antiarrhythmics such as quinine, quinidine, sotalol or amiodarone.

mIsK was enhanced by increasing intracellular Ca²⁺ and by microinjected Ca²⁺–calmodulin dependent protein kinase II. These stimulations were reversed by the calmodulin antagonist W7. Conversely, the phorbol ester PMA, the diacylglycerol analog OAG and microinjected purified protein kinase C inhibited mIsK. This inhibitory effect could be prevented by the protein kinase C inhibitor staurosporine. These results were consistent with the presence of consensus sequences for kinase II and kinase C in the mIsK structure.

Cultured newborn mouse ventricular cardiac cells exhibited a delayed rectifier K⁺ current which had biophysical properties similar to those of cloned mIsK and which was inhibited by clofilium and protein kinase C activators.

In situ hybridization experiments revealed that mIsK mRNA was homogeneously distributed in the cardiac tissue. Neonatal mouse heart expressed the most mIsK mRNA compared with various other rat and mouse tissues. Since this K⁺ channel generates a current which appears to be involved in the control of both the action potential duration and the beating rate, these results suggest an important role for the mIsK channel in cardiac cell physiology and cardiac pathology.

Key words: antiarrhythmic/clofilium/cDNA/PMA/W7/*Xenopus* oocyte

heart, K⁺ currents are mainly involved in limiting the action potential duration and thus are major determinants of cardiac performance. Several K⁺ channels are known to exist in the heart including transient, delayed rectifier (Noble, 1984), ATP-dependent (Noma, 1983), Ca²⁺-dependent (Siegelbaum and Tsien, 1980) or Na⁺-activated (Kameyama *et al.*, 1984) channels. Recent advances in molecular biology have allowed the cloning of several families of voltage-dependent K⁺ channels in mammals. The available structural information clearly indicates that there are two very different families. The first class of mammalian K⁺ channel genes is homologous to *Drosophila* K⁺ channel genes called *Shaker*, *Shaw*, *Shab* and *Shal* (for a review, see Jan and Jan, 1990). This family includes channels of the delayed rectifier type as well as of the transient type. There are extensive similarities between the structural organization of voltage-dependent K⁺ channels and of the previously cloned voltage-dependent Na⁺ and Ca²⁺ channels (Betz, 1990; Stühmer *et al.*, 1989). Some representatives of this family of voltage-dependent K⁺ channels have been recently cloned in the heart (Tseng-Crank *et al.*, 1990; Roberts and Tamkun, 1991; Tamkun *et al.*, 1991). Their subunit molecular weight is 56–95 kDa. The second type of K⁺ channel structure only includes at the present time one single representative channel called IsK. This K⁺ channel was originally cloned in rat kidney and is mainly found in epithelial cells (Takumi *et al.*, 1988) but it is also present in rat uterus and in neonatal rat heart (Folander *et al.*, 1990; Pragnell *et al.*, 1990). The IsK protein consists of 130 amino acids with a single putative transmembrane domain and its structure differs completely from that of the other known ion channel proteins (Betz, 1990). When expressed in *Xenopus* oocytes, this K⁺ channel current closely resembles the delayed rectifier K⁺ current recorded in ventricular cardiac cells (Hall *et al.*, 1963; Noble and Tsien, 1969; McDonald and Trautwein, 1978; Arena and Kass, 1988; Sanguinetti and Jurkiewicz, 1990). It has a slow voltage-dependent activation and a virtual absence of inactivation. The IsK protein, because of its small size and its single subunit structure, is an attractive system to investigate molecular mechanisms involved in the induction and regulation of voltage-dependent K⁺ channel activity. The present report describes the cloning, the main developmental and distribution properties and the expression in *Xenopus* oocytes of the IsK protein from neonatal mouse heart (mIsK). Because of the importance of K⁺ channels in electrophysiological disorders associated with cardiac ischemia and because K⁺ channels are targets for an important class of antiarrhythmic drugs, the pharmacological properties of this channel have been analyzed. Particular emphasis has been placed on the analysis of the mouse IsK channel regulation by Ca²⁺–calmodulin-dependent protein kinase II and protein kinase C. This electrophysiological investigation was extended to cultured newborn mouse ventricular cardiac cells using patch-clamp experiments.

Introduction

Electrophysiological studies have identified a large number of K⁺ channels which differ in their gating commands, their unitary conductances, their pharmacology and their particular functions (Rudy, 1988; Bernardi *et al.*, 1989). In

Results

Microinjection of newborn mouse cardiac poly(A)⁺ mRNA into *Xenopus* oocytes directed the expression of a large long-lasting outward current which did not undergo inactivation (Figure 1A). The current–voltage relationship of this current displayed a marked outward going rectification when the potential was made more positive than −40 mV (Figure 1A). The amplitude of the outward current recorded after microinjection of poly(A)⁺ mRNA extracted from newborn mouse heart was significantly larger than the current obtained with poly(A)⁺ mRNA extracted from adult mouse heart (Figure 1B). Non-injected or tRNA-injected oocytes did not express this outward current (Figure 1B).

A DNA fragment generated by PCR corresponding to the IsK gene initially cloned from rat kidney (Takumi *et al.*, 1988) was used to probe mRNAs from various mouse and rat tissues by Northern blot analysis. Consistent with electrophysiological results, a strong signal was found in mouse neonatal heart transcripts (Figure 2A). The hybridizing transcript level varied with cardiac development. It dropped with age to a weak but detectable signal at the adult stage. Almost no signal was detected in brain, skeletal muscle or liver, both at the embryonic and adult stages of development (data not shown). In agreement with previous studies (Folander *et al.* 1990), a rather weak signal corresponding to a ~0.9 kb RNA transcript was observed in neonatal rat heart tissue and this hybridizing signal was no longer detectable in 1 week-old and adult rat heart. Conversely, a strong signal was found in adult rat kidney but not in the neonate (Figure 2A). In the mouse kidney, the situation was rather similar to that found with the rat, although the general transcript abundance was lower.

Fig. 1. A. Microinjection of poly(A)⁺ mRNAs isolated from newborn mouse heart into *Xenopus* oocytes directs the expression of outward currents. The holding potential was −80 mV and the oocyte was depolarized to −60 mV, −40 mV, −20 mV, 0 mV and +20 mV. The I–V curve was constructed after subtracting the linear leakage. B. Amplitude of the currents recorded after a 40 s depolarization to +20 mV from a holding potential of −80 mV in non-injected, poly(A)⁺ mRNA from newborn mouse heart and from adult mouse heart injected oocytes.

The mIsK was cloned from a neonatal mouse heart cDNA library. Sequence analysis of this cDNA clone (Figure 2B) showed that it encodes a putative protein of 129 amino acids which is 92.3% identical with the rat IsK (Takumi *et al.*,

Fig. 2. Nucleotide and deduced amino acid sequences, Northern blot analysis and cardiac distribution of the mouse IsK clone.

A. Expression of mouse and rat IsK mRNAs in heart and kidney. Poly(A)⁺ mRNAs isolated from heart (4 μ g) and kidney (12 μ g) of neonatal, 1 week-old and adult mice and rats were resolved by electrophoresis, blotted onto nylon membranes and probed with the rat IsK DNA probe as described in Materials and methods. Autoradiograms were exposed for 20 h. B. mIsK cDNA sequence and deduced amino acid sequence of its coding protein. The position of the putative transmembrane domain is indicated below the nucleotide sequence. ∇ , potential N-glycosylation sites; $\star$, serine residue within a consensus Ca²⁺–calmodulin-dependent protein kinase II recognition site; $\bullet$, serine residue within a consensus protein kinase C recognition site. C. Specific expression of mIsK in mouse heart. Poly(A)⁺ mRNAs (5 μ g) from neonatal and adult mouse hearts were electrophoresed, blotted to nylon membranes and probed with the specific mIsK cDNA probe as described in Materials and methods. The autoradiogram was exposed for 20 h. D. Distribution of mIsK RNA in neonatal (1 week-old) and adult mouse heart sections were prepared and hybridized as described in Materials and methods.

1988) and 76% identical with the human counterparts (Murai *et al.*, 1989). The mouse IsK protein has essentially the same structural characteristics as its human and rat IsK equivalents. These include a single putative transmembrane domain surrounded by charged amino acid residues, two potential N-glycosylation sites (residues 5 and 26) at the amino-terminal portion and a single cysteine (residue 106) at the carboxy-terminal portion. The mouse IsK protein sequence indicates the existence of a Ca²⁺-calmodulin-dependent protein kinase II consensus site (Kemp and Pearson, 1990) at Ser40 and a consensus sequence for protein kinase C phosphorylation (Woodgett *et al.*, 1986) at Ser 68.

Figure 2C shows a Northern blot analysis of neonatal and adult mouse heart mRNA probed with the mouse cDNA clone. In neonatal cardiac tissue, the probe hybridized strongly to two main bands at ~0.9 kb and 3.4 kb. Our

recent results (manuscript in preparation) indicate that the large 3.4 kb mRNA species encodes an identical protein coding sequence extended to a further downstream 3' flanking region which results from an alternative use of polyadenylation sites. Although less abundant, the 0.9 kb and 3.4 kb mRNA species were still detectable in adult mouse cardiac tissue. Two additional high molecular weight bands (~6 and 7 kb) were clearly detected in neonatal mouse heart. Heterogeneous mRNA species have also been observed for the rat IsK protein (Iwai *et al.*, 1990). The mIsK cDNA probe binds to a single gene on genomic Southern blots under conditions of high stringency (not shown).

In situ hybridization histochemistry (Figure 2D) indicates that mIsK mRNA is highly expressed in virtually all types of neonatal mouse cardiac cells, including those of the atria and ventricles. Nevertheless, these *in situ* hybridization data do not provide single cell resolution and certainly do not necessarily include cells of the specialized conduction system. The uniform distribution seen at the neonatal stage was similarly observed in adult heart. However, the *in situ* hybridization signal was much weaker in adult heart. This result is in agreement with Northern blot analyses.

cRNA microinjection led to the expression of large outward currents with the same general characteristics as those found for the expression of neonatal heart poly(A)⁺ mRNA. The amplitude of the outward current measured at +20 mV was 983 ± 49 nA ($n = 47$). The biophysical properties of mIsK have been investigated in detail, as shown in Figure 3. The I-V curve (Figure 3) shows that the IsK current rectified in the outward direction and that the activation threshold was -40 mV ($n = 16$). The ionic mechanisms underlying this outward current were then examined by determining the current-voltage relationship. In control conditions (2 mM K⁺), the reversal potential was estimated to be about -100 mV. When the electrochemical gradient for K⁺ was decreased by raising the external K⁺ concentration to 21 mM, the reversal potential was -40 mV which is close to the expected value from the Nernst equation.

Fig. 3. Expression of the mIsK clone in *Xenopus* oocytes. Cells were injected with 10 ng of *in vitro* transcribed RNA (cRNA) encoding the channel and 2 days later were screened for the expression of K⁺ currents in the presence of 300 μ M SITS to block endogenous Ca²⁺-dependent chloride channels. **A.** Currents elicited by a 40 s depolarization step from a holding potential of -80 mV to +40 mV in 10 mV increments. Membrane potentials are indicated on the current traces. Current-voltage relationship for cRNA (filled circle) and non-injected oocytes (open circle). **B.** Estimation of the reversal potential of mIsK using tail currents. The oocyte was first depolarized for 30 s to +20 mV from a holding potential of -80 mV and then repolarized to various potentials as indicated on the current traces. Current-voltage relationships of tail currents are represented in the presence of 2 mM K⁺ (open circle) and 21 mM K⁺ (filled circle) in the external medium. The reversal potential was observed at about -100 mV in the presence of 2 mM K⁺, which is close to the equilibrium potential of K⁺ ions in *Xenopus* oocytes. When the K⁺ concentration in the external medium was increased to 21 mM, the reversal potential was shifted to -40 mV, i.e. the value expected from the Nernst equation. **C.** Voltage dependence of mIsK activation. Superimposed tail current traces obtained when 30 s depolarizing steps were applied from a holding potential of -80 mV. The pulse duration was sufficient for nearly complete activation of mIsK at the potentials tested. Tail currents were recorded at -40 mV. The time course of the tail currents could be fitted by the sum of two exponentials (data not shown). The voltage dependence of the conductance was obtained from the initial amplitude of the tail currents and normalized to the peak tail current. The continuous line is the best fit obtained using a Boltzmann relation with half-activation at -11.3 mV and a slope factor of 9.4 mV. **D.** The onset of mIsK activation was described by the sum of two exponentials. The trace fit and the experimental data were superimposed, demonstrating an accurate fitting procedure. Time constants used for fitting the data were τ_1 : 3411 ms, τ_2 : 24514 ms (R : 0.999). The holding potential was -80 mV and the oocyte was depolarized to 20 mV.

Fig. 4. Pharmacological characterization of mIsK. Inhibition of mIsK by 30 mM tetraethylammonium (**A**) and by 5 mM Ba²⁺ (**B**). The holding potential was -80 mV and oocytes were depolarized to 20 mV every 60 s. **C.** Inhibition of mIsK with increasing concentrations of clofilium. Concentrations used are indicated near each current trace. **D.** Current-voltage relationships of mIsK in control conditions in the presence of 100 μ M clofilium and after the addition of 5 mM Ba²⁺.

These results indicate that mIsK expressed in *Xenopus* oocytes is carried selectively by K^+ ions. Figure 3C shows analysis of the activation parameters. The half-point activation was -11.3 mV and the slope factor was 9.4 mV. Figure 3D shows that the onset of mIsK activation followed a time course described by the sum of two exponentials.

mIsK activity was reduced by the classical, non-selective, K^+ channel blocker tetraethylammonium (30 mM) and by Ba^{2+} (5 mM) (Figure 4A and B). 4-Aminopyridine (3 mM), another voltage-dependent K^+ channel blocker, had no effect (data not shown; $n = 4$). mIsK was also insensitive to dendrotoxin I (10 nM), apamin (100 nM), β -bungarotoxin (10 nM), MCD peptide (100 nM), charybdotoxin (50 nM), quinine (300 μ M) and quinidine (300 μ M). Class III antiarrhythmics (Cook and Quast, 1989) such as amiodarone, sotalol, bretylium tosylate and tedisamil at a concentration of 300 μ M did not affect mIsK ($n = 4$). However mIsK was blocked in a dose-dependent manner by clofilium (Figure 4C). Half maximum inhibition was observed at ~ 100 μ M clofilium ($n = 4$). Current–voltage relationships presented in Figure 4D show that clofilium shifted the activation threshold of the IsK channel by ~ 20 mV in the positive direction.

Because of the previously described consensus sequence at Ser 40, Figure 5 analyzes the regulation of mIsK by Ca^{2+} and more specifically by a Ca^{2+} –calmodulin kinase process. Microinjection of Ca^{2+} –calmodulin-dependent kinase in an oocyte expressing the cRNA for mIsK consistently increased the amplitude of mIsK (Figure 5A). Figure 5B shows that the potentiation of mIsK activity by

protein kinase II microinjection could be reversed by the calmodulin antagonist W7 (10 μ M). mIsK activity was also increased by $\sim 30\%$ in the presence of the Ca^{2+} ionophore A23187 (1 μ M) (Figure 5C). This increase in mIsK amplitude was again inhibited by 10 μ M W7. The intensity of the mIsK current was increased in the presence of high external Ca^{2+} concentration (10 mM instead of 1.8 mM) (Figure 5D). This effect could again be reversed by W7 (10 μ M). The current–voltage relationships illustrated in Figure 5D show that an increase in extracellular Ca^{2+} increased the amplitude of mIsK without modifying the voltage threshold for activation. Treatment of cRNA-injected oocytes with 1 mM 8-Br-cAMP, 3 μ M forskolin or 1 mM 8-Br-cGMP had no effect on mIsK ($n = 4$; data not shown).

Figure 6A shows that the protein kinase C activator phorbol-12-myristate-13-acetate (PMA) used at 30 nM was a potent inhibitor of mIsK. The current–voltage relationship in Figure 6A indicates that PMA treatment induced a 10 mV shift of the voltage threshold for activation in the positive direction. The remaining current after PMA treatment could be fully inhibited with 100 μ M clofilium (Figure 6A). The phorbol ester 4α -phorbol-12,13-didecanoate (PDA) which does not activate protein kinase C had no effect on mIsK activity at a concentration as high as 1 μ M ($n = 5$), although in the same oocyte 0.1 μ M PMA could inhibit mIsK in the same way as described in Figure 6A. PMA had no effect on the K^+ channel activity when oocytes had been pretreated for 30 min with 0.1 μ M staurosporine or 10 μ M H7, two compounds known to inhibit protein kinase C ($n = 4$; Figure 6C). Inhibition of mIsK activity was also observed after treatment with the

Fig. 5. mIsK regulation by Ca^{2+} -dependent processes. **A.** Intracellular microinjection of purified protein kinase II both enhanced the mIsK current and the tail current amplitudes. **B.** The increase in mIsK amplitude due to intracellular microinjection of purified kinase II is reversed by the calmodulin antagonist W7 (10 μ M). **C.** Effect of the Ca^{2+} ionophore A23187 (1 μ M) on mIsK activity. This experiment was performed in the presence of 300 μ M SITS. Addition of 10 μ M W7 reversed the effect of A23187. **D.** Effect of increasing extracellular Ca^{2+} concentration on mIsK activity. In this experiment, the chloride channels were not blocked by SITS. Therefore in the presence of 10 mM external Ca^{2+} , the amplitude of the initial transient outward Ca^{2+} -activated chloride current was enhanced. Activation of mIsK was reversed by 10 μ M W7 although the chloride current remained unchanged. Current–voltage relationships of mIsK (open circle) in the presence of 1.8 mM Ca^{2+} and in the presence of 10 mM Ca^{2+} (filled circle). In A, the holding potential was -60 mV and the oocytes were depolarized to 20 mV. In B, C and D the holding potential was -80 mV and the oocytes were depolarized to $+20$ mV every 60 s.

Fig. 6. mIsK regulation by protein kinase C. **A.** Effect of the protein kinase C activator PMA (30 nM). PMA was superfused for 25 min. The remaining current in the presence of PMA was fully inhibited with 100 μ M clofilium. Current–voltage relationship in control conditions, in the presence of 0.1 μ M PMA and in the presence of both 0.1 μ M PMA and 100 μ M clofilium. **B.** Effect of the synthetic diacylglycerol OAG (100 μ M). OAG was superfused for 15 min. **C.** Comparative effects of PMA (0.1 μ M) and of the 4α -derivative PDA (1 μ M), which does not activate protein kinase C. Antagonism by staurosporine (0.1 μ M) of the PMA-induced inhibition of mIsK (filled circle). **D.** Microinjection of purified protein kinase C depressed mIsK. Further addition of 30 nM PMA enhanced mIsK-induced inhibition. In A, B and C, the holding potential was -80 mV and cells were depolarized to 20 mV for 40 s every 60 s. In D the oocyte was depolarized to 40 mV.

synthetic diacylglycerol OAG ($n = 4$; Figure 6B). Finally, the intracellular microinjection of purified protein kinase C also depressed mIsK (Figure 6D). The addition of 30 nM PMA in the presence of kinase C produced a more extensive inhibition of mIsK (Figure 6D).

Electrophysiological experiments with isolated ventricular cardiac cells from newborn mouse are presented in Figure 7. They show that clofilium (100 μ M) decreased the beating rate, increased action potential duration and enhanced contraction (Figure 7A). Voltage-clamp experiments with the same cardiac cells in culture indicate that clofilium blocked a delayed outward current (Figure 7B). The time course of this delayed rectifier outward current in mouse cardiac cells was slow but substantially faster than the time course of IsK after expression in oocytes (Figures 1A and 3A). Current–voltage relationships obtained by voltage-clamp in cardiac cells were comparable to those of mIsK in oocytes (Figure 3A). PMA (10 nM) consistently depressed the delayed rectifier current recorded in single newborn mouse ventricular cardiac cells (Figure 7C). This effect of the phorbol ester was similar to effects described in Figure 6.

Discussion

There are probably more than 10 different types of K⁺ channels in mammalian cardiac cells. Many of them are voltage-dependent and/or activated via G protein processes (Brown, 1990); others are sensitive to variations of intracellular concentrations of Ca²⁺, ATP, Na⁺ or arachidonic acid (Siegelbaum and Tsien, 1980; Noma, 1983; Kameyama *et al.*, 1984; Kim and Clapham, 1989).

It is particularly interesting to observe that poly(A)⁺

mRNA from newborn mouse heart, when injected into *Xenopus* oocytes, essentially expressed one type of slowly activating K⁺ channel, which did not inactivate and which displayed an outward going rectification. The corresponding channel has been cloned and expressed. It displayed the same voltage-dependence and the same time course as the channel expressed by total poly(A)⁺ mRNA. This strongly suggests that the 129 amino acid protein which has been cloned and which is a close parent of the epithelial channel, initially cloned from rat kidney, constitutes the major voltage-dependent K⁺ channel of mouse heart cells at this stage of development. Electrophysiological results, Northern blot analyses and *in situ* hybridization analysis show that although the level of expression of this IsK channel decreased during cardiac development, this type of K⁺ channel is still expressed in the adult cardiac cell but at a much lower level. At all stages of development the channel seems to be homogeneously distributed in the cardiac tissue.

Oocyte expression of the cRNA for mIsK has allowed a detailed analysis of some of the biophysical and pharmacological properties of this K⁺ channel. The current was inhibited by high tetraethylammonium and Ba²⁺ concentrations but was resistant to a wide variety of other blockers of voltage- or Ca²⁺-dependent K⁺ channels such as 4-aminopyridine, quinine or quinidine, or the different high affinity polypeptide toxin inhibitors such as apamin, MCD peptide, dendrotoxin I, β -bungarotoxin and charybdotoxin. The channel was not affected by classical class III antiarrhythmics (Cook and Quast, 1989) such as amiodarone, sotalol, bretylium tosylate or tedisamil. However, it was strongly inhibited by clofilium (at concentrations in the 10–100 μ M range) which also belongs to the category of class III antiarrhythmics. Therefore it is probable that class III antiarrhythmics, although they all block delayed outward K⁺ channels, discriminate between different types of voltage-dependent K⁺ channels. This view would be in agreement with the recent observations using a sotalol analog, E-4031, that there could exist two different components of the delayed rectifier K⁺ current in guinea pig ventricular myocytes (Sanguinetti and Jurkiewicz, 1990). The drug-sensitive K⁺ current in this preparation activates more rapidly than the E-4031-insensitive component which may correspond to the mIsK type of channel described in this work. The rapid component could correspond to the other class of K⁺ channel structures with subunits of 56–95 kDa in molecular weight.

The channel activity appears to be controlled both by a Ca²⁺–calmodulin-dependent protein kinase process and by protein kinase C. Increases of internal Ca²⁺ or injection of Ca²⁺–calmodulin-dependent protein kinase II increased the mIsK current and all these effects could be abolished by the calmodulin antagonist W7. These observations suggest that this mIsK channel is not only a voltage-dependent K⁺ channel but also a Ca²⁺-sensitive channel. One possible interpretation of these results would be a phosphorylation by kinase II at Ser40 (Figure 2) in the mIsK structure. Such an activation by Ca²⁺ would probably occur after ischemia and reperfusion, when it is known that the cardiac cell is loaded with Ca²⁺.

This paper provides strong evidence for a potent negative regulation by kinase C. Inhibition of mIsK activity has been observed after intra-oocyte injection of kinase C but also after activation of the endogenous oocyte kinase C with the phorbol ester PMA or with the synthetic diacylglycerol

Fig. 7. The delayed rectifier K⁺ current in newborn mouse ventricular cardiac cells maintained in primary culture. **A.** The effects of clofilium were investigated in spontaneously beating heart cells. Superimposed traces of action potentials recorded with an intracellular microelectrode (upper traces) and contractions (lower traces) recorded with an image analyzer in control conditions and after 100 μ M clofilium.

B. Current–voltage relationship of the delayed outward K⁺ current recorded in a single cell with the tight seal patch-clamp technique. The holding potential was -60 mV and the cell was depolarized every 30 s. The delayed K⁺ current was blocked in the presence of 100 μ M clofilium. Both the onset of the delayed rectifier current and the deactivation current were fitted by single exponentials (data not shown) ($R > 0.99$); τ activation = 805 ms at a membrane potential of 20 mV. **C.** The delayed rectifier K⁺ current recorded with the tight seal patch-clamp technique in a single newborn mouse cardiac cell maintained in primary culture was depressed with 10 nM PMA. The holding potential was -60 mV and the cell was depolarized to 20 mV.

OAG. Staurosporine, a kinase C inhibitor, prevented PMA effects. Modulation of mIsK activity by kinase C could occur on Ser68 (see Figure 2). Stimulation of α_1 -adrenergic receptors is known to result in action potential prolongation and inotropic responses in various cardiac preparations (Mukherjee *et al.*, 1983). Since activation of α_1 -adrenergic receptors produces diacylglycerol and therefore triggers kinase C activation, an inhibition of mIsK could contribute to the modulation of action potential and contractile properties. Since the consensus sites for phosphorylation by Ca^{2+} -calmodulin-dependent kinase II and by protein kinase C are located on both ends of the putative transmembrane region domain (Figure 2B), the observed regulation by both kinases would indicate that the amino- and carboxy-termini are cytoplasmic. It should then be assumed that the transmembrane segment crosses the membrane twice as recently proposed for the SS1–SS2 regions of another class of voltage-sensitive K^+ channel (Stevens, 1991). However, it should be remembered that this work cannot definitely establish whether the modulatory effects of kinases (in both oocyte experiments and in mouse cardiac cells) involves a direct phosphorylation of the channel protein (the most probable hypothesis) or a phosphorylation of distinct channel-associated protein(s).

cAMP-dependent processes have been previously shown to be important in the regulation of cardiac excitability. They play an important role in the regulation of L-type Ca^{2+} channel activity (Osterrieder *et al.*, 1982; Reuter, 1984) in relation to the activation of β -adrenergic receptors. They have also been proposed to be involved in the activation of voltage-dependent K^+ channels of the delayed rectifier type (Walsh and Kass, 1988). The IsK sequence contains no consensus site for cAMP-dependent kinase and no effect of 8-Br-cAMP or of forskolin (a stimulator of intracellular cAMP production) could be observed. Therefore the mIsK channel is not a direct target of cAMP-dependent kinase but, one cannot eliminate an indirect regulation of IsK by cAMP. If there are two different components in the delayed rectifier K^+ current observed in cardiac cells, it is possible that only one of the components is sensitive to cAMP regulation. The IsK sequence contains no consensus sequence for cGMP-dependent kinase and no effect of 8-Br-cGMP could be observed.

It was of course important to check whether neonatal mouse cardiac cells express this channel. Three criteria were used to identify this mIsK channel in cultured cells: (i) biophysical properties, (ii) sensitivity to clofilium and (iii) inhibition by PMA. A K^+ channel was indeed present in cultured neonatal ventricular cardiac cells with a slow activation, no inactivation, the same voltage dependence as the cloned mIsK and inhibited by both clofilium and PMA. It is interesting to observe, however, that the channel was substantially faster in the cardiac cells than in the oocyte after poly(A)⁺ mRNA or cRNA injection. This is not an unusual observation since voltage-dependent Ca^{2+} channels are also slower when expressed in oocytes (Dascal *et al.*, 1986) than in their original cell type. Clofilium attenuation of the K^+ channel activity in the mouse cardiac cells in culture increased action potential duration (and therefore Ca^{2+} entry during the plateau phase), increased contraction and had bradycardic effects on the spontaneously beating cells.

The molecular characterization of the IsK channel now permits the search for more potent and possibly specific blockers which obviously would be interesting as potentially

new antiarrhythmic agents. Also, since cardiac hypertrophy often leads to the re-expression or higher degree of expression of proteins which were predominant at the pre- or neonatal stage, it is of a particular interest to examine the status of this mIsK channel in hypertrophic hearts.

Materials and methods

RNA isolation and analysis

Total cellular RNA was extracted (Chomczynski and Sacchi, 1987) and poly(A)⁺ RNA isolated (Aviv and Leder, 1972). For Northern blot analysis, aliquots of poly(A)⁺ RNA were resolved by electrophoresis through a 1 M formaldehyde–1.2% agarose gel and blotted onto Hybond-N membranes (Amersham) in 20 × SSC (3 M sodium chloride, 0.3 M sodium citrate, pH = 7.0). The rat IsK DNA probe used for probing Northern blots and for screening the cDNA library was obtained by the polymerase chain reaction (PCR) using primer sequences corresponding to the previously described rat kidney IsK channel (Takumi *et al.*, 1988). The mouse IsK cDNA probe was the *Xba*I–*Ban*I restriction fragment. DNA fragments were labelled with [α -³²P]dCTP (> 10⁹ c.p.m. per μ g DNA) by reaction with the Klenow fragment of DNA polymerase using random hexanucleotides as primers (Feinberg and Vogelstein, 1983). The blots were hybridized to the probes (1.5 × 10⁶ c.p.m./ml) in 50% formamide, 5 × SSPE (0.9 M sodium chloride, 50 mM sodium phosphate, pH 7.4, 5 mM EDTA), 0.1% SDS, 5 × Denhardt's solution, 20 mM potassium phosphate (pH 6.5) and 250 μ g/ml denatured salmon sperm DNA at 42°C (rat IsK probe) or 55°C (mouse IsK probe) for 18 h. Blots were washed stepwise to a final stringency of 0.1 × SSC, 0.1% SDS at 55°C.

Isolation of cDNA clone encoding mIsK

An oligo(dT) primed cDNA library, derived from poly(A)⁺ mRNA isolated from newborn mouse hearts was constructed by cloning into the *Eco*RI site of phage λ ZAPII (Stratagene). Double-stranded cDNA, prepared with a cDNA synthesis kit (Amersham) was methylated by *Eco*RI methylase, blunted by T4 DNA polymerase and ligated to an *Eco*RI linker (Boehringer). After *Eco*RI digestion, the cDNA was passed through a Sepharose CL-4B column. Fractions longer than ~0.4 kb were pooled and ligated into the vector. Recombinant phages were screened by plaque hybridization with the rat IsK DNA probe in 50% formamide, 5 × SSC, 4 × Denhardt's solution, 0.1% SDS, 100 μ g/ml denatured salmon sperm DNA at 37°C for 18 h. Filters were washed stepwise to a final stringency of 0.1 × SSC, 0.1% SDS at 50°C. Five hybridization positive clones were isolated from about 8 × 10⁴ clones. The λ ZAPII containing the cDNA insert was converted to plasmid cDNA by rescue excision (Stratagene). The cDNA insert (C₁₁₁₁ clone) was subjected to restriction enzyme analysis and DNA sequencing on both strands by the dideoxy nucleotide chain termination method (Sanger *et al.*, 1977) using T7 DNA polymerase (Pharmacia).

Synthesis of cRNA and in situ hybridization

For *Xenopus* oocyte injection, a single-stranded cRNA sense was synthesized using T7 RNA polymerase (RNA transcription kit from Stratagene) and the mIsK cDNA clone linearized by *Sac*I. *In situ* hybridization was performed as described previously (Séquier *et al.*, 1990). Briefly, the mice were deeply anaesthetized and perfused transcardially with 0.9% NaCl followed by ice-cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS, pH 7.4). The hearts were removed and post-fixed in the same solution for 2 h, then washed overnight at 4°C with gentle agitation in a 20% sucrose/PBS solution. They were frozen on dry ice and kept at –70°C. 12 μ m cryostat sections on 3-aminopropyltriethoxysilane-coated slides were used. The tissue sections were hybridized directly with a RNA probe complementary to the mIsK gene, labelled with [α -³⁵S]UTP (1000 Ci/mmol, Amersham). The specific activity of the probes was routinely between 10⁷ and 10⁸ c.p.m./ μ g DNA. Pretreatment of sections was omitted. The hybridization buffer contained 4 × SSC, 50% formamide, 2 × Denhardt's solution, 1% N-laurylsarcosine, 0.12 M phosphate buffer at pH 7.2, 10% dextran sulfate and 10 mM 2-mercaptoethanol. Each slide was incubated with 25 μ l of hybridization solution containing ~3 × 10⁵ c.p.m. of probe (5 ng). Hybridizations were performed for 16 h at 55°C in a humidified chamber. Following hybridization, sections were washed in 1 × SSC, 10 mM 2-mercaptoethanol first at room temperature twice for 30 min, then at 65°C twice for 45 min. Slides were dehydrated and air-dried prior to placing under Amersham β -max Hyperfilm. After appropriate autoradiography, sections were dipped in Ilford K5 photographic emulsion diluted 1:1 with distilled water and exposed for 3–4 weeks at 4°C. Dipped slides were developed for 4 min in Kodak D19 developer and

fixed in Kodak Unifix. All slides were counterstained with haematoxylin/eosin. Control experiments were performed to ensure the specificity of the *in situ* hybridization signal with a sense orientation probe radiolabelled to the same specific activity.

mRNA injection into *Xenopus* oocytes

Xenopus laevis were purchased from C.R.B.M. (Montpellier, France). Pieces of the ovary were surgically removed and individual oocytes were dissected away in a modified Barth's solution: 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄; 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES); pH 7.4 with NaOH. To discard follicular cells, Stage V and VI oocytes were treated for 2 h with collagenase (1 mg/ml, Boehringer) in Barth's medium. 10 ng RNA were injected per oocyte using a pressure microinjector. Oocytes were then kept for 2–7 days in Barth's medium supplemented with 100 UI/ml penicillin and 100 µg/ml streptomycin.

Electrophysiological measurements

In a 0.3 ml perfusion chamber, a single oocyte was impaled with two standard glass microelectrodes (0.5–2.0 MΩ resistance) filled with 3 M KCl and maintained under voltage clamp using a Dagan 8500 amplifier. Stimulation of the preparation, data acquisition and analyses were performed using pClamp software (Axon Instruments; USA). Drugs were applied either externally by addition to the superfusate (Gilson peristaltic pump; flow rate: 3 ml/min) or internally by pressure injection (Inject+Matic; Switzerland) using an additional micropipette (4–6 µm in diameter). The injected volume was 0.01–0.02% of the cell volume. Cytoplasmic protein kinase C was purified to homogeneity from bovine brain according to Walton *et al.* (1987) and microinjected into oocytes at a pipette concentration of 10 µg/ml. Purified Ca²⁺-calmodulin-dependent protein kinase II (Kuret and Schulman, 1984) was microinjected at 10 µg/ml in the presence of 50 µM calmodulin (pipette concentration). A saline solution (ND 96) of the following composition was used in all procedures unless otherwise stated: 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 2 mM MgCl₂, 5 mM HEPES (pH calibrated to 7.4 with NaOH). Leakage currents were digitally subtracted by pulsing to hyperpolarizing potentials and then subtracting a linearly scaled inverted pulse from the depolarization-induced current records. The variability of the results was expressed as the standard error of the mean with *n* indicating the number of cells contributing to the mean. The Student *t* test was used for statistical analysis (*P* < 0.01).

Single cardiac cell experiments

Primary cultures of single ventricular cardiac cells from newborn mice were prepared as previously described for rat ventricular cells (Shubeita *et al.*, 1990). Culture dishes were used immediately after the replacement of the culture medium by the specific solution for electrophysiological recordings.

The whole cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was used to record ionic currents. The external solution at pH 7.4 contained 140 mM tetramethylammonium (TMA) chloride, 1 mM CaCl₂, 2 mM MgCl₂, 5 mM KCl, 5 mM glucose, 10 mM HEPES/TMAOH. The pipette solution contained 140 mM potassium gluconate, 2 mM MgCl₂ and 2 mM EGTA. This solution was buffered at pH 7.3 with 10 mM HEPES-KOH. Pipettes (2–5 MΩ) were connected to the headstage of the patch clamp amplifier (RK 300, Bio-Logic, France). For action potential recordings, the external medium at pH 7.4 contained 140 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM KCl, 5 mM glucose, 10 mM HEPES-NaOH. Intracellular recordings were made using conventional glass microelectrodes filled with 3M KCl, with resistance of 20–50 MΩ. The microelectrode was connected to an electrometer preamplifier (WPI M707) allowing simultaneous injection of current and voltage recording through the same microelectrode.

The mechanical activity was recorded simultaneously with the electrical activity (Romey *et al.*, 1988) using a 128 × 128 photodiode array matrix associated with an image analyzer giving an image rate analysis up 200/s.

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