J. Membrane Biol. 188, 127–135 (2002)
DOI: 10.1007/s00232-001-0180-4

The Journal of
Membrane
Biology

Characterization of a Ca^{2+}-activated Nonselective Cation Channel during Dedifferentiation of Cultured Rat Ventricular Cardiomyocytes

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Received: 22 December 2001 / Revised: 28 March 2002

Abstract. Cardiac hypertrophy is associated with electrical activity modifications, including sustained depolarization, that lead to a propensity for arrhythmias. The ionic currents underlying the sustained depolarization are not well defined. Similar modifications were reported on adult rat cardiomyocytes in primary culture undergoing dedifferentiation. Using the single-channel measurements on these cells, we identified the appearance of a Ca^{2+}-activated nonselective cation channel (NSC_{Ca}) during the dedifferentiation process. In excised inside-out patches the channel presented a linear I/V relationship with a conductance of 26.5 pS. It was equally selective for Na^+ and K^+ and impermeable to Cl^- and Ca^{2+} ions. The open probability increased with depolarization and with rise in intracellular calcium concentration. The channel activity was reduced by intracellular ATP and suppressed by flufenamic acid. Channel detection increased after incubation with a purinergic receptor agonist (ATPγS) or a PKC activator (PMA). Furthermore, occurrence of the channel developed during the culture. Absent at one day in vitro (*d.i.v.*), channel activity was present in 5, 46, 27 and 19% of patches after 4, 7, 14 and 21 *d.i.v.*, respectively. We suggest that the channel may be associated with pro-arrhythmic signaling, in particular during the release of transmitters from autonomic nerve endings in the hypertrophied hearts.

Key words: Cation channel — Dedifferentiation — Cardiomyocyte — Arrhythmia

Introduction

Myocardial hypertrophy is an adaptive response to chronic pressure overload that enables the heart to maintain cardiac function. However, in the long term, cardiac hypertrophy contributes to sudden death from arrhythmias (Levy et al., 1987; Kannel & Cobb, 1992). The appearance of abnormal automaticity is the result of remodeling that occurs in both the myocyte and interstitial compartments of the heart. During the remodeling, reintroduction of a fetal program has been reported, which consists of the functional expression of a number of proteins. This process induces changes in intracellular calcium, contractile proteins, ion channels, the adrenergic system, the extracellular matrix, cardiac autocrine functions and electrophysiological properties. The main electrophysiological changes associated with myocardial hypertrophy are a significant prolongation of the action potential accompanied by a sustained depolarization, which may contribute to arrhythmic activity (Kleiman & Houser, 1988; Gomez et al., 1997). The ionic basis of these electrophysiological modifications has been linked mainly to: 1) a reduction in the transient outward current (I_{to}) and in the inward rectifier current (I_{K1}) and 2) a re-expression of both pacemaker channels, the low voltage-activated calcium channel ($I_{Ca,T}$) and the hyperpolarization-activated "funny" channel (I_f) (for review *see* Swynghedauw, 1999).

Although the depolarization of the resting membrane potential is consistent with the modifications of pacemaker currents (I_f and I_{CaT}) and background potassium current (I_{K1}), expression and changes in other ionic channels may also contribute to the altered action potential configuration. In the cardiomyocytes a number of nonselective cationic channels (NSC) have been described. They are spontaneously active or activated in pathological

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conditions associated with a rise in intracellular calcium concentration, a decrease in intracellular ATP or stretch (Carmeliet, 1999). These currents are considered background currents that can be implicated in the genesis of arrhythmias.

As reported by Hefti et al. (1997), primary culture of adult myocytes is considered a myocardial hypertrophy model. The cardiomyocytes maintained in serum-containing medium undergo drastic morphological remodeling from the elongated in vivo structure to a spherical flat shape with numerous extensions, and they resume spontaneous rhythmic beating (Jacobson & Piper 1986; Eppenberger et al., 1988, 1994; Fares, Gomez & Potreau 1996; Veldkamp, de Jonge & van Ginneken, 1999). In this model, the early program of gene expression is reactivated for several genes. Significant accumulation of the re-expressed proteins, e.g., β-MHC (Nag & Cheng, 1986; Eppenberger et al., 1988), α-sm actin (Eppenberger-Eberhart et al., 1990), ANF (Eppenberger-Eberhart et al., 1993, 1997) and both pacemaker currents I_f and I_{CaT} (Farès et al., 1996, 1998) can be demonstrated after several days in culture. The initial breakdown and reassembly of myofibrils and regaining of contractile function, together with the accompanying molecular changes, recapitulate the events associated with hypertrophy in vivo (Schaub et al., 1997). Cardiomyocytes in primary culture thus present a suitable in vitro model for detailed analysis of the hypertrophic reaction at the cellular level, particularly to investigate the mechanisms underlying regulation of ion channel functions.

The purpose of our study was to characterize the presence of a nonselective cation channel during the process of cellular remodeling observed in the primary culture of ventricular myocytes isolated from rat heart. Here, we report the detection of a calcium-activated nonselective cation channel (NSC_{Ca}) in dedifferentiated cardiac ventricular myocytes. Using the inside-out configuration of the patch-clamp technique, we analyzed its electrophysiological properties: selectivity, unitary conductance, voltage-dependence and regulation.

Materials and Methods

Cell Isolation and Culture

Rat ventricular myocytes were obtained as previously described (Farès et al., 1996). All experiments were performed according to national ethical guidelines (French Ministry of Agriculture). Briefly, adult (250–300 g) male Wistar rats were injected with 1000 i.u. heparin I.P. (Choay; Sanofi, Gentilly, France) and anesthetized with ether; the heart was quickly removed via thoracotomy under aseptic conditions and transferred to an ice-cold Tyrode solution. The aorta was cannulated and the heart mounted on a Langendorff apparatus, then successively perfused (at 37°C) with the following oxygenated solutions: 4 min with Tyrode solution to recover its spontaneous activity; 4 min with a nominally Ca^{2+}-free Tyrode solution, and 15 to 20 min with the same solution supplemented with 100 i.u. ml^{-1} collagenase (type II, Worthington), 0.06 mmol/l $CaCl_2$ and 0.1% bovine serum albumin (BSA). Once flaccid, the heart was rinsed 2 min without collagenase. Ventricles were cut off and stored in Kraft-Brühe (KB) medium (Isenberg & Klockner, 1982), finely minced with iridectomy scissors and gently triturated using a fire-polished Pasteur pipette. Isolated cells were filtered to remove undissociated pieces, maintained 30 min in KB medium and gradually resuspended in the culture medium: M199 medium (Gibco, Life Technologies) supplemented with 10^{-7} mol/l insulin, 0.2% BSA, 10 % fetal calf serum (Boehringer Mannheim), 1% antibiotics (penicillin, 100 i.u. ml^{-1}; streptomycin, 50 i.u. ml^{-1}; Sigma) and 10 μmol/l cytosine 1-β-D arabinofuranoside (Sigma), an inhibitor of fibroblast proliferation. Seeded into 35-mm Petri dishes pretreated with laminin (20 mmol/l; Sigma), cells were incubated for 2 hr at 37°C for plating. The culture medium was changed once, then renewed every 2 days while dishes were kept at 37°C with a constant moist 95% air –5% CO_2 environment. During the culture, the morphology of cardiomyocytes undergoes large modifications and is clearly different from that of the cardiac fibroblasts. The freshly isolated rod-shaped myocytes were attached to the substrate and progressed to a rounded shape; after 1 week, most of the cells were largely flattened and considerably bigger with apparent multinucleation, and they showed spontaneous activity.

Solutions and Chemical Products

For cell dissociation, the Tyrode solution contained (in mmol/l): 140 NaCl; 5.4 KCl; 1.8 $MgCl_2$; 1.8 $CaCl_2$; 10 glucose; 10 HEPES; pH was adjusted to 7.35 with NaOH.

For electrophysiological recordings, the standard solution (140 mmol/l NaCl solution) for bath and pipette contained (in mmol/l): 140 NaCl; 4.8 KCl; 1.2 $MgCl_2$; 10 glucose; 10 HEPES. Pipette solution contained 1 mmol/l $CaCl_2$, while bath solution contained 2 mmol/l EGTA reducing the calcium concentration to 1 nmol/l. The use of a low concentration of calcium in the bath minimizes myocyte contractions, which make the seal formation difficult, particularly on the dedifferentiated cells. Low NaCl solution contained 14 mmol/l NaCl (no KCl included) and was supplemented with 256 mmol/l sucrose to maintain osmolarity. To test the monovalent cation selectivity, 140 mmol/l NaCl was replaced by KCl. Ca^{2+} permeability was determined using a solution containing (in mmol/l): 100 $CaCl_2$, 10 glucose, 10 HEPES. External solutions (pipette and bath) were adjusted to pH 7.4. The pH of perfused solutions (inside of the membrane) was adjusted to 7.2. Internal Ca^{2+} concentrations below 10 μmol/l were determined with a combination of $CaCl_2$ and Ca-EGTA buffers (Teulon, Paulais & Bouthier, 1987).

Prior to the seal, cells were incubated for at least 10 min in a solution containing 140 mmol/l NaCl for the control or 100 μmol/l adenosine 5′-0-3-thiotriphosphate (ATPγS) or 200 μmol/l 8-(4-chlorophenylthio) adenosine cyclic monophosphate (cpt-cAMP), 1 mmol/l 3-isobutyl-1-methylxanthine (IBMX) and 20 μmol/l forskolin (referred as the cAMP-mixture) or 500 nmol/l phorbol 12-myristate 13-acetate (PMA). PMA was previously dissolved in DMSO with a final ratio for DMSO of 0.1% that had no effect on channel activation ($n = 10$). Chemical products were from Sigma.

Measurements

Single-channel currents were recorded under voltage clamp with an RK400 (Biologic, Claix, France) patch-clamp amplifier from patches of cardiomyocytes in culture, using the inside-out variant of the patch-clamp technique (Hamill et al., 1981). Patch pipettes

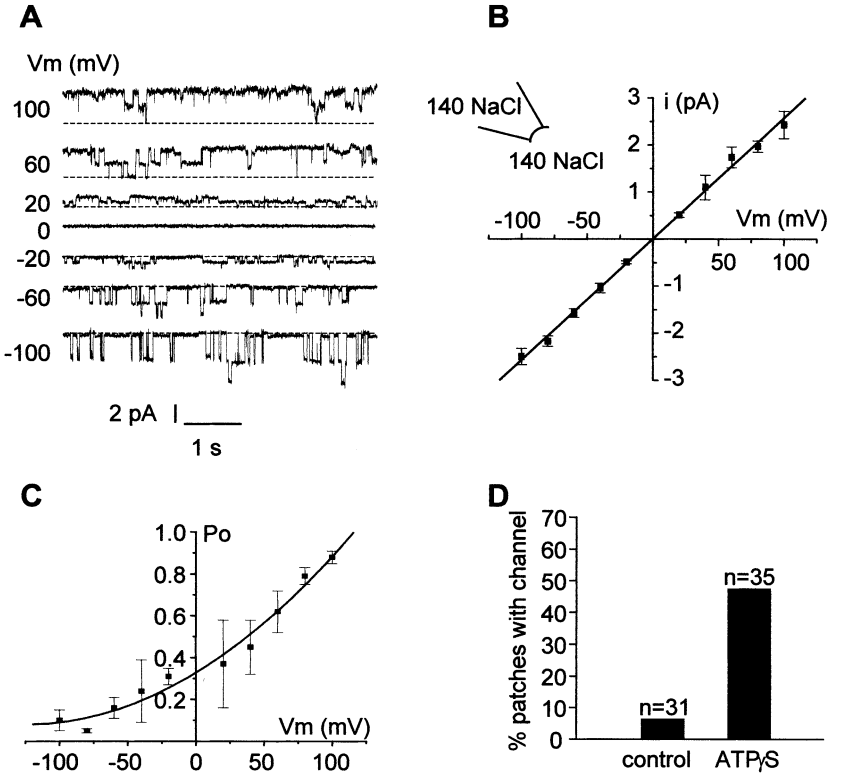

Fig. 1. Conductive properties of the channel in dedifferentiated ventricular myocytes. (*A*) Single-channel current tracings recorded at various voltages from an inside-out patch at 8 *d.i.v.* Pipette and bath: 140 mmol/l NaCl. In all figures, V_m is the applied membrane potential and dotted lines indicate the current level of closed channels. (*B*) Corresponding current-voltage relationship. The data points (means ± SEM, $n = 4$–5) are fitted to a straight line ($g = 26.5$ pS; $E_R = -0.3$ mV). (*C*) Voltage dependence of open probability (*Po*). In four experiments similar to that shown in *A*, *Po* is determined at various voltages using amplitude histograms. (*D*) Occurrence of the channel in control cells and cells pre-incubated with ATPγS. Experiments were done on dedifferentiated cells at 7–8 *d.i.v.; n* denotes the number of cells.

with a tip resistance of 7–10 MΩ (in 140 mmol/l NaCl-solution) were made from microhematocrit glass tubes and coated with Sylgard (Dow Corning, Seneffe, Belgium). The signal displayed on an oscilloscope (HM205-3, Hameg, Germany) was stored on a DAT recorder (DTR 1204, Biologic). The bath reference was an Ag/AgCl pellet. Liquid junction potentials arising from changes in bathing solutions at the inner surface of the membrane patch were determined with the standard pipette solution (140 mmol/l NaCl). In presence of 145 mmol/l KCl external solution, the junction potential was −2 mV. Junction potentials were −9.2 and +9 mV in presence of 14 mmol/l NaCl and 100 $CaCl_2$ mmol/l, respectively. These values are compatible with those calculated by the JPCalc program (Barry, 1994) from the Clampex software version 8.1 (Axon Instruments, Foster City, CA). The applied potentials ($V_m = V_{bath} - V_{pipette}$) were corrected accordingly. Currents due to the migration of cations from the inner to the outer surface of the membrane were positive and were registered as upward deflections in single-channel current tracings. All experiments were conducted at room temperature.

Data Analysis

Signals for analysis were subsequently played back and filtered (300 Hz) through a model 902LPF eight-pole Bessel filter (Frequency Devices, Haverhill, MA) and digitized at 1 kHz using a Digidata 1200A analog-digital interface and Fetchex software version 6.02 (Axon Instruments). Single-channel current recordings were then analyzed with Bio-patch software version 3.30 (Biologic). Computer analysis generated amplitude histograms for construction of *I/V* curves and estimates of the open probability (*Po*). Relative permeabilities were deduced from reversal potentials (E_r) for current flows obtained after fitting *I/V* curves using the Goldman-Hodgkin-Katz (G-H-K) equation (Origine 5.0 Software). Experimental values were given as mean ± SEM. Comparisons between groups were done using Student's *t*-test.

Results

A nonselective cationic channel (NSC) has been characterized, for the first time, in neonatal cardiomyocytes in culture by Colquhoun et al. (1981). The authors observed a NSC channel in inside-out patches with equimolar Na concentration (140 mmol/l) on the intra- and extracellular side.

According to their results and in similar conditions, single-channel currents were recorded from dedifferentiated cardiomyocytes (at least 7 days of culture) to characterize a possible functional expression or activation of nonselective channels during the cellular remodeling process. Figure 1*A* illustrates a channel activity recorded after application of 1 mmol/l calcium into the bath. Reapplication of a Ca^{2+}-free solution suppressed the channel activity (*not shown*). The calcium-activated current has a linear current-voltage (*I/V*) relationship and a reversal potential of −0.3 ± 2.8 mV. The slope conductance value is 26.5 pS ± 1.3 pS ($n = 5$) (Fig. 1*B*). The open-probability values determined for various membrane potentials clearly indicate that channel activity increases with depolarization (Fig. 1*C*). In the present experimental conditions, channel openings were detected only in 6.4% of patches ($n = 31$). The activity of the calcium-dependent cation channel recorded in dedifferentiated cardiomyocytes was similar to that observed in plasma membrane of various excitable and non-excitable mammalian cells, in particular in neonatal cardiomyocytes (Colquhoun

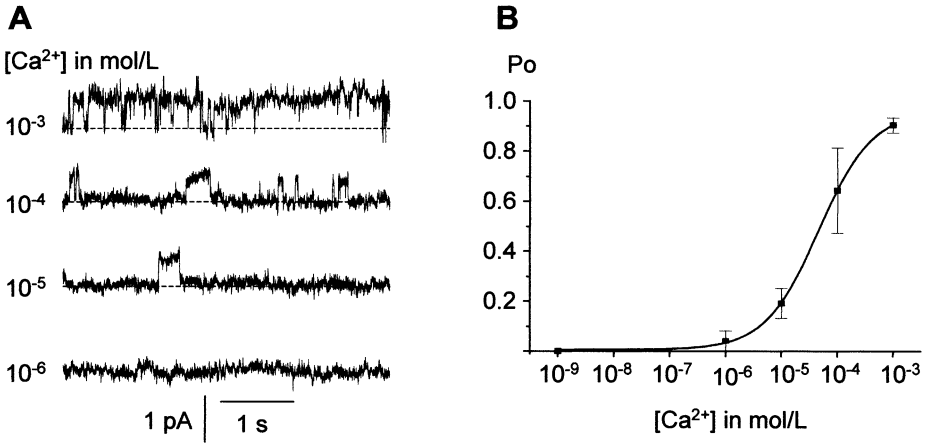

Fig. 2. Effect of internal calcium on channel open probability. (*A*) Single-channel currents recorded from an inside-out patch showing that channel activity increases with rise in $[Ca^{2+}]$. V_m = 30 mV. Pipette and bath: 140 mmol/l NaCl. Cell at 9 *d.i.v.* The internal $[Ca^{2+}]$ below 10^{-5} mol/l is determined using EGTA. (*B*) Mean values of *Po* at various $[Ca^{2+}]$. Bars are means ± SEM of 4–6 patches for each concentration.

et al., 1981) and adult cardiac ventricular cells (Ehara, Noma & Ono, 1988). The NSC channel has been also described in outer hair cells of mammalian cochlea (Van den Abbeele, Tran Ba Huy & Teulon, 1994, 1996). In this preparation, the channel is activated and regulated by the stimulation of purinergic receptors, which induces an increase in intracellular calcium concentration via a pathway involving the phospholipase C (PLC) cascade. As similar regulation of purinergic receptors has been reported in the heart (for review, *see* Vassort, 2001), a poorly hydrolyzable ATP analog (ATPγS), known as an agonist of purinergic receptors, was tested on the dedifferentiated myocytes. Before patching, cells were incubated at least 10 minutes with 100 μmol/l ATPγS. Excised inside-out membrane patch-clamp experiments were performed at various voltages in symmetrical ionic conditions (140 mmol/l NaCl / 140 mmol/l NaCl) with 1 mmol/l $CaCl_2$ added to the bath. In these conditions, the electrophysiological properties of the calcium-activated channel were similar to those obtained in untreated cells. However, the frequency of channel activity recordings was strongly increased (45.7% *vs.* 6.4 in control; *see* Figure 1*D*). For that reason, the following studies on the ionic selectivity of the channel and its modulation by calcium and intracellular ATP were undertaken on ATPγS-treated cells.

Effect of Internal Calcium

As activation of the channel needs the presence of intracellular calcium, the effect of different Ca^{2+} concentrations at the cytoplasmic side was studied in inside-out patches. Figure 2*A* shows that increasing calcium concentration from 1 μmol/l to 1 mmol/l reversibly enhances channel activity. The open probability of active channels determined at +30 mV is plotted in Fig. 2*B* as a function of the Ca^{2+} concentration in the bath. Channel openings were seen at $[Ca^{2+}]_i > 1$ μM. At 1 mmol/l $[Ca^{2+}]_i$, the open probability was 0.85 ± 0.05. Fitting experimental data points by the Hill equation yields an apparent dissociation constant (*K*) at 56.7 ± 8.6 μmol/l and the Hill coefficient is estimated to be 0.99 ± 0.13.

Selectivity Properties of the NSC Channel

The ionic selectivity of the channel was studied in inside-out patches by changing the ionic composition of the bath solution. As shown in Fig. 3*A* and *B*, reduction of NaCl from 140 to 14 mmol/l at the internal side of the membrane (*circles*) caused a pronounced inward rectification and shifted the reversal potential (E_r) to more positive voltages. The shift value is 40 ± 4.1 mV (n = 6) compared to the I/V curve obtained in symmetrical 140 mmol/l NaCl conditions (Fig. 1*B*). The value of channel conductance measured in the negative voltage range does not change (21.7 ± 3.1 pS). When NaCl in the bath is replaced by KCl (Fig. 3*B*, filled squares), the channel conductance of 24 ± 1.7 pS and the E_r of 0.5 ± 5.7 mV (n = 4) are not significantly different from control values. The permeability ratios P_{Cl}/P_{Na} and P_K/P_{Na} are, respectively, 0.09 and 0.98. In these ionic conditions, potassium channels were also recorded in some cells. Contrary to the NSC channels, these potassium channels can be observed at every stage of the cultures. To test NSC channel permeability for calcium, the inside of the membrane was bathed in the solution containing 100 mmol/l $CaCl_2$. In these conditions, the channel displayed a conductance of 18.6 ± 0.5 pS, with a reversal potential of 48 ± 3.5 mV (n = 6), corresponding to a P_{Ca}/P_{Na} of 0.09 ± 0.01 (Figs. 3*C* and *D*). The results indicate that the channel primarily conducts monovalent Na^+ as well as K^+ ions, but is not permeable to Ca^{2+} or Cl^- ions.

Effect of Internal ATP

Nonselective cationic channels were also shown to be sensitive to intracellular adenine nucleotides (Teulon,

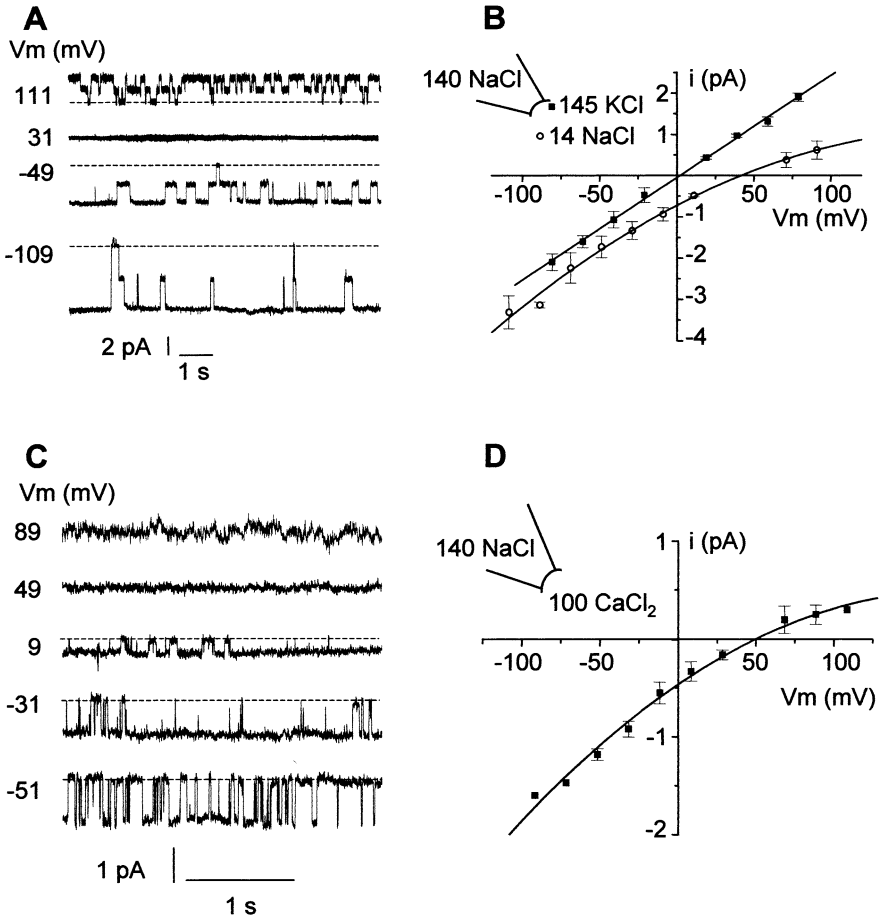

Fig. 3. Selectivity properties of the channel. (*A*) Single-channel current tracings recorded at various voltages from an inside-out patch. Pipette : 140 mmol/l NaCl, bath: 14 mmol/l NaCl. Cell at 21 *d.i.v.* (*B*) Current-voltage relationships of the channel in various ionic conditions. Pipette 140 mmol/l NaCl, bath: 14 mmol/l NaCl (○) as in *A*, or 145 mmol/l KCl (■). The measuring points were fitted satisfactorily with the G-H-K equation for the 14 mmol/l NaCl solution and linear regression for the 145 mmol/l KCl solution. E_R values are 40 mV ($n = 6$) and 0.5 mV ($n = 4$), respectively. $P_{Cl}/P_{Na} = 0.09$ and $P_K/P_{Na} = 0.98$. (*C*) Single-channel current tracings recorded at various voltages from an inside-out patch with pipette 140 mmol/l NaCl and bath 100 mmol/l $CaCl_2$. Cell at 9 *d.i.v.* (*D*) Current-voltage relationship in the same ionic conditions as in *C* ($n = 6$), fitted with the G-H-K equation. $P_{Ca}/P_{Na} = 0.09$.

2000), Figure 4*A* shows that addition of ATP to the cytosolic surface of inside-out excised patches inhibits channel activity. ATP (0.5 mmol/l) reversibly reduced *Po* by 50% to 100% ($n = 3$).

Inhibitory Effect of Flufenamic Acid

Flufenamic acid, a nonsteroidal anti-inflammatory drug, has been reported to block NSC channels (Gogelein et al., 1990). Figure 4*B* and *C* illustrates the action of this compound on channel activity recorded from an inside-out patch held at −40 mV. The presence of flufenamic acid in the bath clearly suppresses the activity. At 500 μmol/l, open probability is reduced by 95.8 ± 4.25% ($n = 4$).

Occurrence of the NSC Channel

The nonselective cation channel was studied in adult ventricular myocytes during several days of culture in inside-out configuration. To enhance channel detection, cells were pre-incubated in the presence of ATPγS. Patch recordings from cells 1 to 22 days in vitro show that the frequency of channel recordings is correlated with the dedifferentiated state of the cells (Fig. 5). Whereas at days 7 and 8, half of the patches contained NSC channel activity, no channel activity was observed in the majority of patches at day 1. However, the percentage of patches showing channel activity progressively declined after 8 days of culture. Such a decrease can be explained by the re-differentiation process described in the model (Jacobson & Piper, 1986). The single-channel conductance was unchanged with days in culture.

Effects of Protein Kinase Activators

In the heart, it has been reported that ATPγS may stimulate the PKA and/or PKC pathway through purinoreceptors (Legssyer et al., 1988; Puceat et al., 1998). Therefore, the involvement of each kinase pathway in the activation of NSC channel was studied using activators of PKC and PKA. Dedifferentiated cells (at days 7–8) were pre-incubated either in the presence of a mixture containing 200 μmol/l cpt-cAMP, 1 mmol/l IBMX and 20 μmol/l forskolin to activate PKA (cAMP mixture) or in the presence of 500 nmol/l PMA, a phorbol ester reported to activate PKC. As illustrated in Fig. 6, the cAMP mixture does not change the channel detection (1/20 patches) compared to the control (2/31 patches). In contrast,

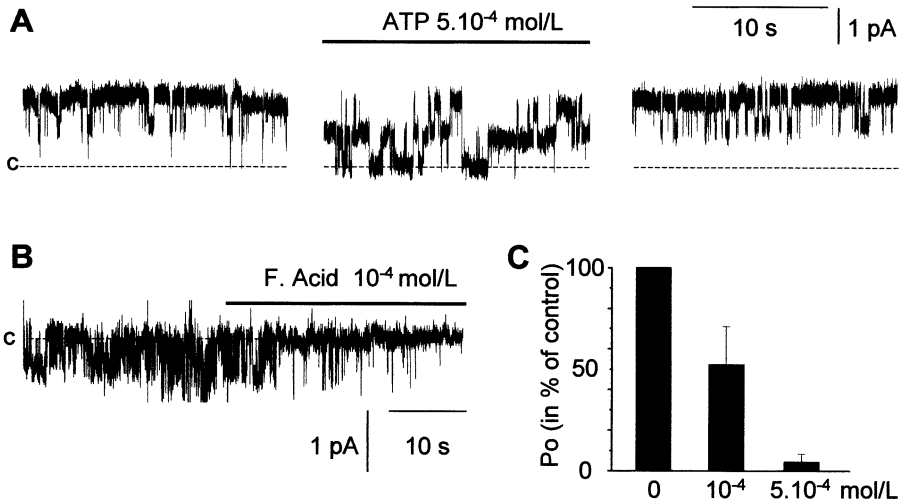

Fig. 4. Pharmacological properties of the channel. (*A*) Single-channel current recorded from an inside-out patch, showing the reversible inhibition of channel activity by 5×10^{-4} mol/l internal ATP. *c* indicates the closed-channel current level. Pipette and bath: 140 mmol/l NaCl, V_m = 40 mV, cell at 8 *d.i.v.* (*B*) Single-channel current recorded from an inside-out patch illustrating the blocking effect of flufenamic acid (*F. Acid*) at 0.1 mmol/l. *c* indicates the closed-channel current level. Pipette and bath 140 mmol/l NaCl, V_m = −40 mV, cell at 9 *d.i.v.* (*C*) Percentage of activity under 0.1 and 0.5 mmol/l flufenamic acid applications, compared with the control before flufenamic acid application. n = 5 and 4, respectively.

as was observed in ATPγS-treated cells, the detection is enhanced after PMA incubation (20/29 patches). This PMA effect is not observed in freshly isolated cardiomyocytes (n = 24) (*not shown*). The results suggest that the channel regulation by purinergic receptor may be mediated by the activation of endogenous PKC.

Discussion

The present study shows the existence of a Ca^{2+}-activated nonselective cation channel in dedifferentiated adult rat ventricular myocytes in primary culture. This channel has properties similar to Ca^{2+}-activated NSC channels present in epithelia, neurons, cardiac tissues, exocrine tissues and cells from sensory organs. In all these cell types, the common characteristics of NSC channels are voltage dependence, single-channel conductance between 20 and 35 pS, lack of discrimination between monovalent cations, low permeability to Ca^{2+}, sensitivity to intracellular calcium ions, inhibition by cytosolic ATP and blockade by flufenamic acid (for review, *see* Teulon, 2000). These properties differ from those of other kinds of NSC channels, such as TRP channels (Harteneck, Plant & Schultz, 2000), serotonin-gated cation channels (Yang, 1990) and mechano-sensitive cation channels (Yang & Sachs, 1993).

In the present work, the pre-incubation by a purinergic receptor agonist (ATPγS) or a phorbol ester treatment (PMA) similarly increased the number of active channels. Direct evidence for extracel-

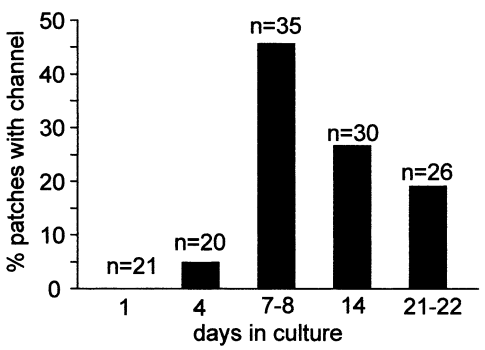

Fig. 5. Percentage (%) of patches exhibiting channel activity with a conductance of 26 pS at different days of primary culture. Cardiomyocytes are pre-incubated with ATPγS. *n* denotes the number of trials for each batch.

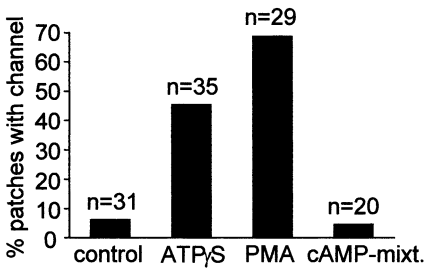

Fig. 6. Percentage of patches exhibiting NSC_{Ca} channel activity after pre-incubation with protein kinase activators PMA 500 nmol/l or cAMP-mixture containing cpt-cAMP 200 μmol/l, IBMX 1 mmol/l, and forskoline 20 μmol/l on cells at 7–8 *d.i.v.* For easier comparison, results from control and ATPγS pre-incubated cells, already presented on Figure 1*D*, are added to the histogram, *n* denotes the number of trials for each batch.

lular ATP-induced increase in PKC activity has been previously observed in cardiac myocytes (Legssayer et al., 1988; Kunapuli & Daniel, 1998). According to these results, PKC could be a second messenger for the purinergic-receptor regulation of NSC channel activity in dedifferentiated cardiomyocytes. The large rate of channel activity observed in inside-out configuration after few minutes of ATPγS treatment suggests that PKC phosphorylation facilitates the functional channel expression. Such a facilitation has been reported in *Xenopus* oocytes, where the translocation of cloned GABA-transporter proteins from the transgolgi complex to the plasma membrane was regulated by PKC-dependent phosphorylation (Corey et al., 1994). Also, direct phosphorylation of the nonfunctional NSC channels leading to an increase in the number of functional channels, cannot be excluded. The implication of cAMP-dependent phosphorylation seems to be excluded since no increase in channel detection is observed on dedifferentiated cells pre-incubated with PKA activators (cAMP mixture). Partridge et al. (1990) have reported that in neurons external application of forskolin or IBMX or membrane cAMP analogs reduced NSC current. Similar reduction induced by PKA has been observed on outer hair cells (Van den Abbeele et al., 1996). A downregulation by PKA cannot be excluded in the present data; although the channel detection rate was not significantly different from the control, the NSC current was recorded only in 1/20 patches after cAMP-mixture application at days 7 and 8 of culture.

In the present study, the channel activity was rarely observed in freshly isolated cardiomyocytes. In freshly dissociated guinea-pig ventricular cells, Ehara et al. (1988) have previously mentioned a very low density of NSC channels with similar properties: they are voltage-dependent, calcium-activated, equally permeable to Na^+ and K^+, and they have a conductance of about 28 pS. However, in the present experiments, the frequency of NSC current recordings is increased with time of culture. It is maximal at days 7 and 8 of culture where the myocytes are strongly dedifferentiated, and in this case is very similar to that usually observed in neonatal rat cardiomyocytes using a fetal gene program (Colquhoun et al., 1981). Thus, the increase in channel-rate recordings observed between 1 and 8 days could be explained by a re-expression of fetal NSC channel gene leading to an increase in the number of functional channels. Such an upregulation during the culture of adult ventricular myocytes has been already reported for several genes such as β-myosin heavy chain (Nag & Cheng, 1986; Eppenberger et al., 1988), atrial natriuretic factor (Eppenberger-Eberhardt et al., 1993, 1997), pacemaker channels I_f and $I_{ca,T}$ (Fares et al., 1996, 1998). Moreover, the background potassium current (I_{K_1}) is significantly reduced and cells exhibit a spontaneous activity characterized by the development of early afterdepolarizations (Veldkamp et al., 1999).

Jacobson, Banfalvi & Schwarzfeld (1985) have reported changes in the value of the resting membrane potential of rat cardiomyocytes in primary culture. Freshly isolated cells have a resting membrane potential of −80 mV. They depolarize during the first week of culture and reach a potential value close to −25 mV at day 7. Then, the membrane potential returns to negative values when cells redifferentiate, to reach −53 mV between 12 and 28 days, and −70 mV at day 60. In a previous study, we have also reported similar variations (Fares et al., 1998). Interestingly, the present study shows that the rate of channel detection is correlated with these changes of membrane potential during primary culture.

Membrane depolarization has been observed in cardiac cells under various pathological conditions and has been attributed to a transient inward current, I_{ti} (Kass, Tsien & Weingart, 1978; Thandroyen et al., 1991). I_{ti} should be due to an intracellular calcium oscillation that depolarizes the cell membrane, generating a delayed after-depolarization. The identity of I_{ti} is controversial, but it appears to be the result of different ionic currents, depending on the experimental conditions. These currents are the electrogenic Na/Ca exchanger, a Ca^{2+}-activated chloride current and a Ca^{2+}-activated nonselective cationic current. Whereas the Na/Ca exchanger and the Ca^{2+}-activated Cl^- current participate under physiological conditions, NSC current has only been demonstrated under extreme experimental conditions involving Ca^{2+} concentration overload (Teulon, 2000). As estimated in the present experiments, the channel is activated by calcium in a micromolar range. In isolated ventricular myocytes, extracellular ATP alone does not exert significant electrophysiological modification. Nevertheless, in the presence of drugs known to increase intracellular calcium concentration, extracellular ATP facilitates the induction of afterdepolarization and increases the amplitude of the transient inward current (Song & Belardinelli, 1994). In consequence, it is conceivable that the NSC current recorded in dedifferentiated cardiac cells is associated with pro-arrhythmic signaling, in particular during the release of transmitters from autonomic nerve endings in the hypertrophied heart. In addition, it is plausible that the current can also be activated in cardiac glycoside toxicity and ischemia conditions where the level of intracellular calcium is drastically increased. It has been reported that oxygen-derived free radicals induce a Ca^{2+}-activated nonselective cationic current in ventricular cells of guinea pig (Jabr & Cole, 1993, 1995). The authors suggest that the NSC current may be involved in disorders associated with myocardial injury during the reperfusion after ischemia. On the other hand, the NSC channel is

not activated at physiological levels of ATP. The channel would only be activated when cell metabolism is beginning to be impaired, e.g., by anoxia or the addition of cyanide. The NSC channel may have profound implication for susceptibility to arrhythmias induced by ischemia in hypertrophied or failing hearts.

In conclusion, a Ca^{2+}-activated nonselective cation channel appears to be a potential candidate for the genesis of arrhythmias, particularly during the release of ATP into the extracellular space under pathophysiological conditions. A better understanding of the regulatory processes governing this channel may lead to improved drug targets and therapies.

We thank Chantal Jougla and Françoise Mazin for technical help and Jacques Teulon for helpful comments on this manuscript.

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