Electrophysiological properties of neonatal mouse cardiac myocytes in primary culture

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1. The increasing utility of transgenic mice in molecular studies of the cardiovascular system has motivated us to characterize the ionic currents in neonatal mouse ventricular myocytes.

2. Cell capacitance measurements (30 ± 1 pF, $n = 73$) confirmed visual impressions that neonatal mouse ventricular myocytes in primary culture are considerably smaller than freshly isolated adult ventricular myocytes. With the use of electron microscopy, mitochondria and sarcoplasmic reticulum were found in close association with myofibrils, but transverse tubules were not observed.

3. Action potential durations, measured at 50 and 90% repolarization, were 23 ± 1 and 42 ± 2 ms respectively ($n = 46$). Application of 4-aminopyridine (4-AP; 5 mM) prolonged action potential duration at 50% repolarization by 26 ± 5% ($n = 3$). The brevity of the action potential is explained by the rapid activation of a transient outward K^+ current upon voltage-clamp depolarization to plateau potentials.

4. Potassium currents identified include an inward rectifier, a large 4-AP-sensitive transient outward, a slowly inactivating 4-AP-insensitive outward, a slowly activating delayed rectifier and a small rapidly activating E-4031 (10 μM)-sensitive delayed rectifier K^+ current.

5. Sodium currents (-305 ± 50 pA pF^{-1}, $n = 21$) were recorded in 40 mM Na^+ with Ni^{2+} (1 mM) to block Ca^{2+} currents and with K^+ replaced by Cs^+. The relative insensitivity of the Na^+ current to block by tetrodotoxin ($IC_{50} = 2{\cdot}2 \pm 0{\cdot}3$ μM, $n = 4$) is distinctive of the cardiac Na^+ channel isoform.

6 Nitrendipine-insensitive (10 μM) Ba^{2+} currents elicited during steps from −90 to −30 mV measured -25 ± 5 pA pF^{-1} ($n = 7$, 30 mM Ba^{2+}). Decay of these currents was complete during 180 ms depolarizations, even with Ba^{2+} as the charge carrier. These currents were not present when the holding potential was set at −50 mV. These data support the presence of a low threshold, T-type Ca^{2+} current.

7. The maximal nitrendipine-sensitive L-type Ca^{2+} current density was -10 ± 2 pA pF^{-1} ($n = 8$) in 2 mM Ca^{2+} and -38 ± 5 pA pF^{-1} ($n = 9$) in 30 mM Ba^{2+}. Exposure to isoprenaline (1 μM) resulted in an 82% increase ($n = 3$) in the amplitude of the Ba^{2+} currents elicited at 0 mV.

8. Neonatal mouse cardiac myocytes in primary culture possess surprisingly large inward currents given the brevity of their action potentials. Their small cell size and apparent absence of transverse tubules makes them technically favourable for whole-cell patch clamp studies. For these reasons, this preparation promises to be useful for the phenotypic characterization of transgenic mice.

Over-expression (or selective knock-out) of genes in transgenic animals has begun to provide unique insights into a variety of physiological processes ranging from differentiation to senescence (Grosveld & Kollias, 1992). Although progress is being made in several species, the techniques for creating transgenic animals have become routine only in mice (Babinet, Morello & Renard, 1989). The introduction of foreign genes into the germ line of mice has recently been tailored to express genes specifically in the heart, such as the SV40 large T-antigen oncogene (Field, 1988) and the c-*myc* proto-oncogene (Jackson, Allard, Sreenan, Doss, Bishop & Swain, 1990) driven by the atrial natriuretic factor promoter. Expression of these transgenes leads to altered

cardiomyocyte growth and differentiation. With the development of model systems that exhibit heritable cardiovascular pathology, it will be of interest to study any resultant abnormalities in excitability and to explore the ionic basis for the altered electrophysiology. Despite the potential importance of such approaches, remarkably little is known regarding the electrophysiology or other cellular properties of adult murine heart and even less about the neonate.

Although the basic electrophysiology of mouse heart has not been extensively studied to date, transmembrane action potentials have been recorded in adult mouse papillary muscle and were found to be of short duration (action potential duration to 50% repolarization, $APD_{50} = 5{\cdot}8 \pm 0{\cdot}7$ ms, and $APD_{80} = 26{\cdot}0 \pm 2{\cdot}4$ ms) (Binah, Arieli, Beck, Rosen & Palti, 1987). Perhaps the most rigorously studied ionic conductance in mouse heart is the transient outward K^+ current. Using single-channel recordings, Benndorf (1988*a*) identified three types of K^+ channels, based on their differing slope conductance measurements, which make up the macroscopic transient outward K^+ current in ventricular cells from the adult mouse. The ensemble average currents of the three channels and the whole-cell currents decayed at two rates, an early fast decay phase followed by a very slow decay of current (Benndorf, 1988*a*). To date the only current which has been examined in neonatal mouse heart is the slow delayed rectifier K^+ current (Honore *et al.* 1991). Although recordings of an inward rectifier K^+ current have not been reported in mouse, cDNA encoding an inward rectifier K^+ channel (IRK1) has been isolated and the transcripts are found in abundance in mouse heart (Kubo, Baldwin, Jan & Jan, 1993). The sparsity of information about the ionic currents in wild-type neonatal mouse cardiac myocytes indicates to us that the present characterization represents a necessary first step towards future phenotypic characterization of cardiac excitation in transgenic mice.

We find that neonatal mouse cardiac myocytes are small and lack transverse (T) tubules, thus rendering them favourable for whole-cell patch clamp recordings. These cells express a variety of K^+ channels, as well as surprisingly robust Na^+ and Ca^{2+} currents.

METHODS

Preparation of myocytes

The procedure used to isolate and culture neonatal mouse cardiac myocytes was adapted from commonly used neonatal rat cardiac myocyte preparations (Rogers, Gaa & Allen, 1986). Hearts ($1{\cdot}4 \pm 0{\cdot}1$ mg dry heart weight (g body weight)$^{-1}$, $n = 11$) were aseptically removed from 1–3-day-old mouse pups (CD-1, Charles Rivers, Willmington, MA, USA) immediately following prompt decapitation. (Decapitation is approved by the American Veterinary Association as an acceptable means of killing mouse pups.) All the hearts from one litter (10–18 pups) were pooled, minced and placed in a tube containing a minimal essential medium (MEM)-based digestion solution containing collagenase (see below). At 37 °C, slow rotation of the tube containing the digestion solution and heart tissue sufficed to disperse cells. The supernatant fractions, containing the cells, were collected during repeated collagenase digestions and added to digestion solution (without collagenase) on ice. The cell-containing fractions were centrifuged at low speed, resuspended in digestion solution (with 0·2% fetal bovine serum) and filtered (cell strainer 35-2350, Becton Dickinson & Co., Lincoln Park, NJ, USA). Cells were plated at low density (2×10^5 cells ml^{-1}) in growth medium to ensure the presence of isolated myocytes for electrophysiological study. After plating, the cells were incubated in a 95% O_2–5% CO_2 atmosphere at 37 °C for 1–3 days. Adherent viable myocytes demonstrated spontaneous contractile activity within 12 h of plating. While this contractile activity was not examined in detail, the pattern and frequency of beating in isolated cells was variable and independent of other isolated myocytes in the culture dish. Cessation of spontaneous contractions coincided with the run-in of modified Tyrode solution (wash-out of growth medium) and may also be associated with the equilibration of cells to room temperature from 35 °C. Only cells observed beating in growth medium, prior to the wash-in of Tyrode solution, were selected for whole-cell patch clamp experiments. Initial experiments determined that non-beating cells were inexcitable, presumably because of their depolarized membrane potential.

Solutions

The digestion solution was made from Joklik MEM (Sigma Chemical Co., St Louis, MO, USA) with the following additions (mM unless otherwise given): Hepes, 10; sodium pyruvate, 10; L-glutamine, 5; nicotinamide, 1; L-ascorbate, 0·4; adenosine, 1; D-ribose, 1; $MgCl_2$, 1; taurine, 1; DL-carnitine, 2; potassium bicarbonate, 26; $CaCl_2$, 0·5; gentamycin, 10 μg ml^{-1}; collagenase (Wako Pure Chemical Industries, Richmond, VA, USA), 0·25 mg ml^{-1}; pH, 7·6. The growth medium used for plating and maintenance of cells was composed of Dulbecco's Modified Eagle's Medium (nutrient mixture F-12 HAM, Sigma) with 10% fetal bovine serum and 1% penicillin–streptomycin.

Action potentials were recorded in a modified Tyrode solution composed of (mM): NaCl, 140; KCl, 5·4; glucose, 10; $MgCl_2$, 1; sodium pyruvate, 2; $CaCl_2$, 2; Hepes (Na^+ salt), 10; pH adjusted to 7·4 with NaOH. The 'physiological' pipette filling solution used for recording action potentials contained (mM): KCl, 140; $MgCl_2$, 1; ATP (magnesium salt), 4; NaCl, 10; Hepes, 10; pH adjusted to 7·2 with KOH.

Potassium currents were recorded using a Na^+-free pipette filling solution composed of (mM): KCl, 140; ATP (magnesium salt), 4; EGTA, 5; $MgCl_2$, 1; Hepes, 10; pH adjusted to 7·4 with KOH; while the myocytes were perfused with a Na^+-free solution containing (mM): *N*-methyl-D-glucamine (NMG), 140; KCl, 5·4; glucose, 10; $MgCl_2$, 1; $CaCl_2$, 0·1; $CdCl_2$, 0·5; Hepes, 10; pH adjusted to 7·4 with methanesulphonic acid. The transient outward current blocker 4-aminopyridine (1–5 mM; Sigma) was added directly to the NMG recording solution prior to pH adjustment. The benzenesulphonamide E-4031 (Eisai Co., Tokyo, Japan) was used to identify a rapidly activating component of delayed rectifier K^+ current ($I_{K,r}$) (Sanguinetti & Jurkiewicz, 1990).

Ba^{2+} currents carried through Ca^{2+} channels were recorded in a Na^+-free solution containing (mM): *N*-methyl-D-glucamine (NMG), 100; CsCl, 5; glucose, 10; $MgCl_2$, 1; $BaCl_2$,

30; Hepes, 10; pH adjusted to 7·3 with methanesulphonic acid. Potassium currents were minimized by Cs^+ replacement of K^+. The pipette-filling solution was composed of (mM unless otherwise given): caesium aspartate (obtained by mixing equimolar amounts of L-aspartic acid and CsOH), 135; EGTA, 10; $MgCl_2$, 1; ATP (magnesium salt), 4; cyclic AMP, 100 μM; Hepes, 10; pH adjusted to 7·2 with CsOH. The use of this low-Cl^- internal solution introduced a liquid junction potential of +8 mV. For quantitative comparisons with other studies, the membrane potentials indicated for Ba^{2+} currents (Figs 9–12) should be corrected for this junction potential. Cyclic AMP was excluded from the pipette solution during those experiments which utilized isoprenaline (Sigma) to induce a β-adrenergic response. Nitrendipine (Miles Laboratories, West Haven, CT, USA) was used to block L-type Ca^{2+} channels.

Sodium currents were recorded using a reduced extracellular Na^+ solution containing (mM): tetraethylammonium chloride (TEA-Cl), 100; NaCl, 40; glucose, 10; $MgCl_2$, 1; CsCl, 5; $CaCl_2$, 0·1; $NiCl_2$, 1; Hepes, 10; pH adjusted to 7·3 with CsOH. The pipette-filling solution used to record Na^+ currents was identical to that used in the isoprenaline experiments described above. The sensitivity of the Na^+ current to block by tetrodotoxin (Sigma) was examined with a cumulative concentration–response protocol.

Electrophysiological methods

Cells were studied in the 35 mm plastic culture dishes in which they were plated. All experiments were conducted at room temperature (23–24 °C). Whole-cell transmembrane currents were recorded using the patch clamp method (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) with an integrating amplifier (Axopatch 200A, Axon Instruments, Foster City, CA, USA). Pipettes were made on a Flaming–Brown micropipette puller (model P-87, Sutter Instrument Co., San Rafael, CA, USA) from 1·5 mm o.d. filament-containing glass capillary tubes (World Precision Instruments, Sarasota, FL, USA). After fire polishing, pipettes had tip resistances of 1–3 MΩ when filled with an internal recording solution. Currents were filtered at 2 kHz and digitized with 12-bit resolution.

The adequacy of the voltage clamp was challenged when recording Na^+ currents in excess of 20 nA (Fig. 8) with a 1 MΩ pipette. Series resistance compensation was between 75 and 80% during all experiments. In such experiments, the maximum uncompensated voltage error across the pipette series resistance was calculated to be approximately 5 mV. Under less extreme conditions, for example recording Ba^{2+} currents through Ca^{2+} channels, the maximum voltage error was less than 2 mV.

Cell capacitance was calculated from the capacity current transients elicited by a hyperpolarizing voltage step. The total amount of charge required to change the membrane potential by 10 mV was obtained by integration of the area under the capacity transients. The cell capacitance was obtained by dividing the total amount of charge by the amplitude of the voltage step.

Action potentials were stimulated in current-clamp mode. Whole-cell recording was established in the voltage-clamp mode and compensation for the pipette capacitance was accomplished before switching into the current-clamp mode. Short depolarizing current pulses (0·2–0·3 ms, 100–200 pA) sufficed to initiate action potentials in most cells.

Data acquisition and analysis were performed using custom software. The tetrodotoxin dose–response data was fitted by the following power logistics function (Origin, MicroCal Software, Northampton, MA, USA):

$$\%I_{Na} = (\%I_{Na,i} + \%I_{Na,f})/(1 + ([TTX]/IC_{50})^p) + \%I_{Na,f}.$$

In this equation $\%I_{Na}$ is the fraction of the control Na^+ current remaining at a given concentration of tetrodotoxin, $\%I_{Na,i}$ and $\%I_{Na,f}$ are determined at the lowest and highest concentrations of tetrodotoxin, p is a rate factor, and IC_{50} is the half-blocking concentration of tetrodotoxin. All pooled data are reported as means with standard error of the mean.

Fixation of cells for electron microscopy

Neonatal mouse cardiac myocytes were fixed *in situ* after 1 day in culture with formaldehyde (4%) and glutaraldehyde (1%) in a phosphate-buffered solution for 30 min at room temperature. The cells were post-fixed in osmium tetroxide (1%) for 30 min, dehydrated through graded ethanols (60–100%) and infiltrated with resin (Poly/bed A-12, Polysciences Inc., Warrington, PA, USA). The addition of hardener followed by incubation at 37 °C for 3 days and baking at 60 °C overnight cured the resin. The resin disc containing the cells was separated from the plastic culture dish and thin sections were cut *en face* and examined with an electron microscope.

RESULTS

Morphology

Initial visual impressions that neonatal mouse cardiac myocytes are considerably smaller than freshly dissociated adult ventricular myocytes were substantiated by measurements of capacitance. Mean cell capacitance equalled 30 ± 1 pF ($n = 73$). For comparison, the typical capacitance of adult mammalian ventricular myocytes is 100–200 pF (Benndorf, Boldt & Nilius, 1985; Balke, Rose, Marban & Wier, 1992).

Immediately after dissociation the cells are spherical in appearance. Within hours after plating they develop attachments and become adherent to the bottom of the plastic culture dish. The development of these attachments coincides with flattening and spreading out of the cell. Adherent cells demonstrate spontaneous contractile activity by 12 h in culture. After 1–2 days in culture, the cells are still rather compact in shape, most commonly appearing round or ovoid.

These external morphological changes are accompanied by internal reorganization of myofibrils. Myofibrils, surrounded by mitochondria, were found to be arranged in both normal longitudinal arrays and in star-shaped arrays (Fig. 1*A* and *B*). However, there is still considerable anisotropy, as evidenced by the periodicity of the Z lines. Rings of sarcoplasmic reticulum were observed in circular networks in close association with the arrays of myofibrils. Transverse tubules were not evident in any of the electron micrographs examined, although caveolae (thought to represent T-tubule precursors (Sommer & Johnson, 1968)) were occasionally observed (Fig. 1*B*).

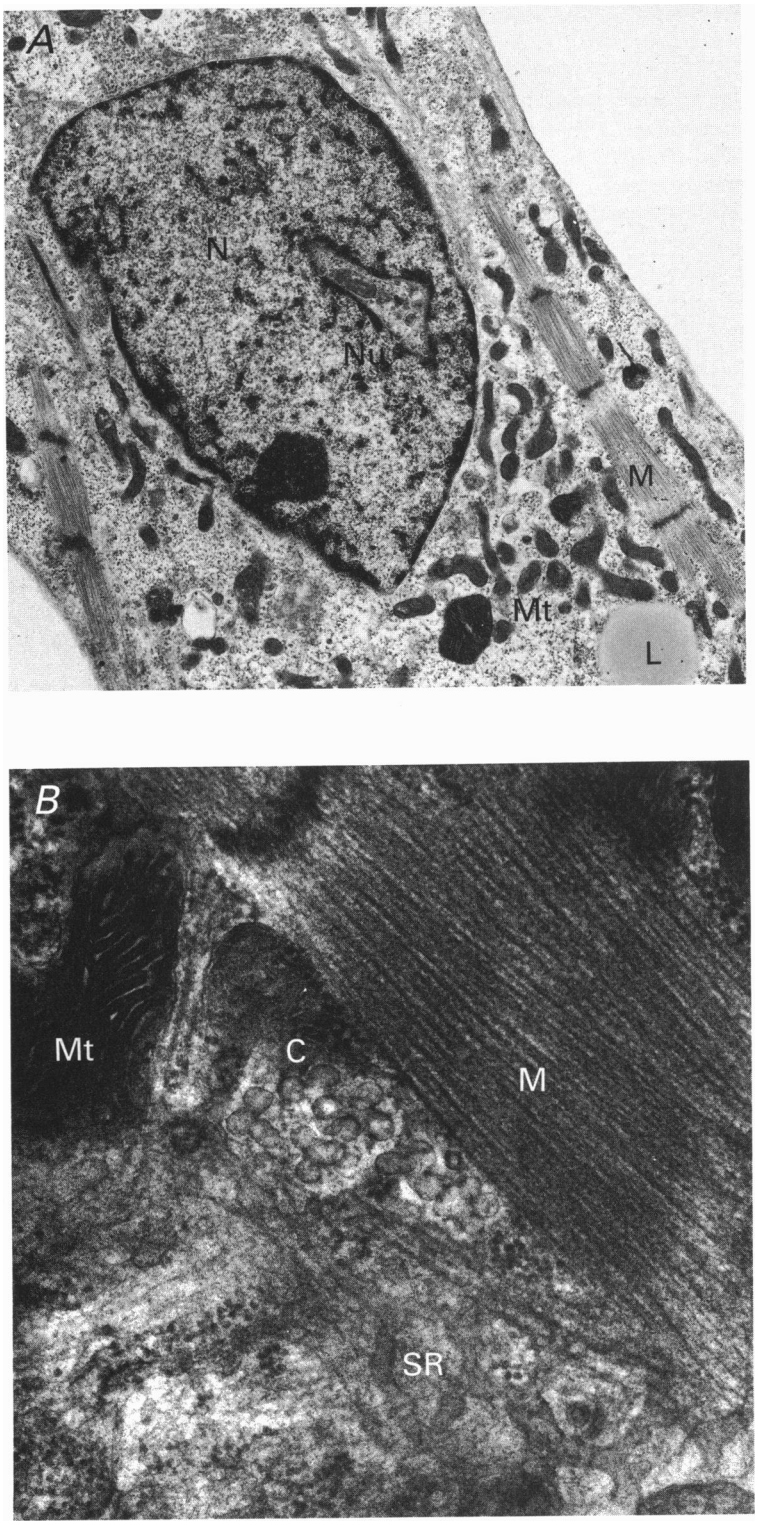

Figure 1. Electron micrographs of neonatal mouse cardiac myocytes
Cells were fixed (see Methods) at day 1 in primary culture. *A* (×6000) illustrates the morphology of the cells when the electrophysiological experiments were performed. Intracellular organelles include a nucleus (N) with prominent nucleolus (Nu), abundant mitochondria (Mt), myofibrils (M) in longitudinal and star-shaped arrays, and lipid droplets (L). *B* is a high-power (×52500) view of a contractile unit with caveolae (C) with circular networks of sarcoplasmic reticulum (SR) in close association with myofibrils. Transverse tubules were not observed in any of the views ($n = 36$) taken of six different cells.

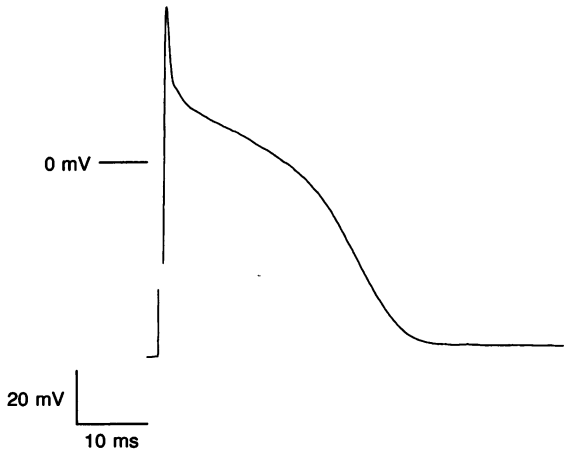

Figure 2. A representative action potential recorded in a neonatal mouse cardiac myocyte bathed in a modified Tyrode solution
The rapid upstroke, overshoot and brief 'plateau' above 0 mV were common features of the action potentials recorded in 46 cells. Resting membrane potential was −82 mV in this myocyte (27 pF).

Action potentials

Action potentials (Fig. 2) recorded in a modified Tyrode solution with 'physiological' pipette solution (see Methods) displayed a rapid upstroke, an overshoot and a brief plateau prior to repolarization. The overshoot of the action potential reached $+34 \pm 1$ mV ($n = 46$). The brevity of the action potential was particularly striking. Action potential durations quantified at 50 and 90% repolarization equalled 23 ± 1 and 42 ± 2 ms ($n = 46$) respectively. Between stimulated action potentials (1 Hz), cells repolarized to a maximum diastolic potential of -83 ± 1 mV ($n = 46$). While there was no evidence of phase 4 depolarization under these ionic conditions in any of the cells studied, the late phase of repolarization evident at the foot of the action potential shown in Fig. 2 was slow, returning to its resting potential over 160–325 ms. In rat ventricular myocytes, this slow phase of repolarization has been attributed to electrogenic Na^+–Ca^{2+} exchange activity removing Ca^{2+} from the cytosol and generating an inward current (Schouten & Ter Keurs, 1985).

Net membrane current

In order to understand the ionic basis for the rapid repolarization of the action potential, net membrane current (Fig. 3*A*) was recorded from a holding potential of −60 mV. The early portion of the total current elicited by weak depolarizations is inward and presumably dominated by Na^+ current. With progressively larger depolarizations the net inward current becomes smaller and quickly gives way to an early outward current. The transition of the early portion of the net membrane current, measured at 20 ms, from the inward to outward direction occurred between +10 and +20 mV (Fig. 3*B*). The magnitude of this early outward current increased with depolarization. The net membrane current elicited at +60 mV displays the characteristics of a transient outward K^+ current (I_{to}) similar to that described in adult rat ventricular myocytes (Josephson, Sanchez-Chapula & Brown, 1984). The predominance of a large I_{to} suffices to explain the rapid repolarization of the action potential.

Potassium currents

In addition to an early outward net membrane current at positive potentials (Fig. 3*A*), prolongation of the action potential duration by 4-aminopyridine (4-AP; Fig. 4 inset; 5 mM) suggested the presence of a 4-AP-sensitive I_{to}, with properties like the calcium-independent I_{to} thoroughly characterized in ferret ventricular myocytes (Campbell, Rasmusson, Qu & Strauss, 1993). Under more rigorous recording conditions, in which K^+ was the only available charge carrier (see Solutions), the voltage dependence of activation and the sensitivity of I_{to} in neonatal mouse myocytes to block by 4-AP was determined by step depolarizations (200 ms) before and during application of 4-AP (2 mM). The 4-AP-sensitive difference currents (Fig. 4*A*) activate very rapidly to a peak and then decay fully during the depolarization. Steps to more positive potentials increase the magnitude of the peak current (Fig. 4*A* and *B*) and speeds up the kinetics of activation and decay of the macroscopic current. In an additional experiment (not shown), I_{to} tail currents were elicited upon repolarization to potentials from −20 to −140 mV after activation of I_{to} by brief depolarizations (10 ms) to +100 mV. The experiment was repeated after the addition of 4-AP to obtain the 4-AP-sensitive I_{to} tail currents. The instantaneous current–voltage relationship of the 4-AP-sensitive I_{to} current reversed at -80 ± 2 mV ($n = 3$, data not shown) which is consistent with a K^+-selective conductance. The predominance of a large I_{to} suffices to explain the rapid repolarization of the action potential.

The 4-AP-insensitive outward current demonstrated little or no time dependence during either short or long step depolarizations. Similarly described time-independent 4-AP-insensitive outward currents have been reduced by replacement of extracellular Cl^- concentration (Campbell *et al.* 1993). The contribution by outward Cl^- currents to this 4-AP-insensitive current should have been minimal in our experiments because the recording solution contained only 8·6 mM Cl^- (reversal potential $E_{rev} = +72$ mV). The current is probably a slowly or non-

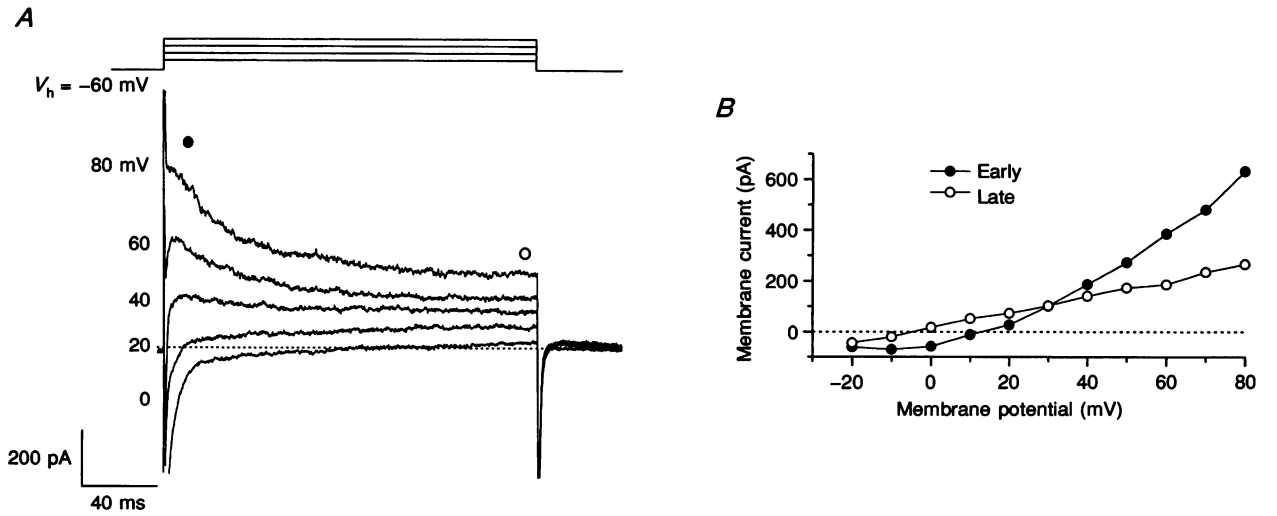

Figure 3. Net membrane currents (*A*) recorded in a modified Tyrode solution and isochronal current–voltage relationships (*B*)

The total membrane current (*A*) evoked by small depolarizations (400 ms) from −60 mV (holding potential, V_h) was dominated by the Na^+ current. Depolarizations to positive potentials elicited outward net membrane currents that activated rapidly and decayed over time with the characteristics of the transient outward current. The total membrane current–voltage relationships (*B*) were taken 20 ms into depolarization (●, early) and just prior to repolarization (○, late). The early portion of the net membrane current is outward at potentials attained during the early phases of the action potential. Net membrane currents with the characteristics of the transient outward current were recorded in 8 other cells. The dotted line in both figures denotes the level of zero current. Cell capacitance, 30 pF.

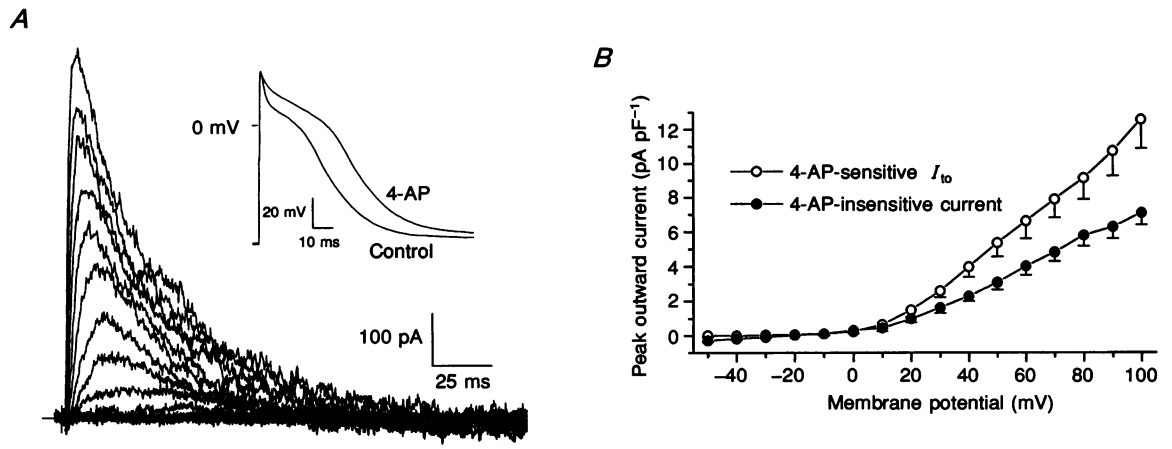

Figure 4. The 4-aminopyridine-sensitive transient outward K^+ current (*A*), the effect of 4-AP (5 mM) on the action potential (inset) and current–voltage relationships for the 4-AP (2 mM) sensitive I_{to} and a 4-AP-insensitive outward current (*B*)

The current records show a family of 4-AP-sensitive I_{to} difference currents obtained by subtracting the 4-AP-insensitive current from the total outward current recorded during 200 ms depolarizations to positive potentials from a holding potential of −60 mV. These currents activated rapidly and displayed inactivation kinetics characteristic of the transient outward current found in adult mammalian ventricle. The current–voltage relationships (*B*, $n = 4$) were obtained by measurements of the peak outward current taken soon after depolarization. Activation of the 4-AP-sensitive I_{to} begins at +10 mV and increases with stronger depolarizations throughout the range of potentials tested. Cell capacitance, 46 pF.

inactivating, 4-AP-insensitive potassium conductance similar to that recorded in neonatal canine ventricular cells (Jeck & Boyden, 1992).

In addition to the transient outward current, inward rectifier and delayed rectifier K^+ currents were identified in neonatal mouse cardiac myocytes. An inward rectifier K^+ current (I_{K1}; Fig. 5*A*) was recorded in normal Tyrode solution in the presence of nitrendipine (10 μM) to block L-type Ca^{2+} current. Hyperpolarizing voltage clamp steps (1 s) from −50 mV (to inactivate Na^+ current and T-type Ca^{2+} current) evoked inward currents. As expected, the current exhibited pronounced inward rectification, with little current at potentials positive to −60 mV. The addition of Ba^{2+} (1 mM) to the recording solution completely abolished I_{K1} (Fig. 5*B*), an effect that was readily reversible (not shown).

Relatively weak depolarizations elicited a delayed rectifier K^+ current in neonatal mouse cardiac myocytes. In these experiments the holding potential was set at −40 mV to inactivate the Na^+ current and T-type Ca^{2+} current. The addition of 4-AP (2 mM) and Cd^{2+} (500 μM) to the recording solution prevented contamination by the 4-AP-sensitive I_{to} and L-type Ca^{2+} currents, respectively. Under these conditions depolarizations (8 s) activated an outward current which grew larger over time, and repolarization evoked outward tail currents which decayed gradually (Fig. 6*A*). The time-dependent current evoked upon depolarization increased with steps to more positive potentials (Fig. 6*B*). Similarly, the amplitude of the tail currents increased with stronger depolarizations (Fig. 6*B*). Honore *et al.* (1991) have cloned a delayed rectifier K^+ channel from a neonatal mouse heart cDNA library (mIsK) which has similar properties to the slowly activating ($I_{K,s}$) delayed rectifier K^+ current (Fig. 6*A*) in neonatal mouse ventricular myocytes.

Rapid run-down of I_K has been reported in whole-cell patch clamp experiments, presumably because equilibration of the intracellular milieu with the pipette solution depletes the cell of requisite intracellular regulatory proteins (Harvey & Hume, 1989). In this preparation, run-down of the slow component of I_K is dramatic, as shown in Fig. 6*C*. The control I_K current was recorded immediately after impalement during a depolarization to +20 mV. After only 4 min the time-dependent $I_{K,s}$ had run down dramatically, yet there was only a small reduction in the tail current amplitude. Test pulses to +20 mV over the next 3 min demonstrated that a fast component of the I_K current was resistant to run-down based on the persistence of the remaining time-dependent current and I_K tail currents. Wash-in of E-4031 (10 μM), a benzenesulphonamide antiarrhythmic agent, eliminated almost all of the time-dependent current and immediately suppressed the I_K tail current. Sanguinetti & Jurkiewicz (1990) identified a rapidly activating component of delayed rectifier K^+ ($I_{K,r}$) in guinea-pig ventricular myocytes by its sensitivity to block by E-4031. We have also identified an E-4031-

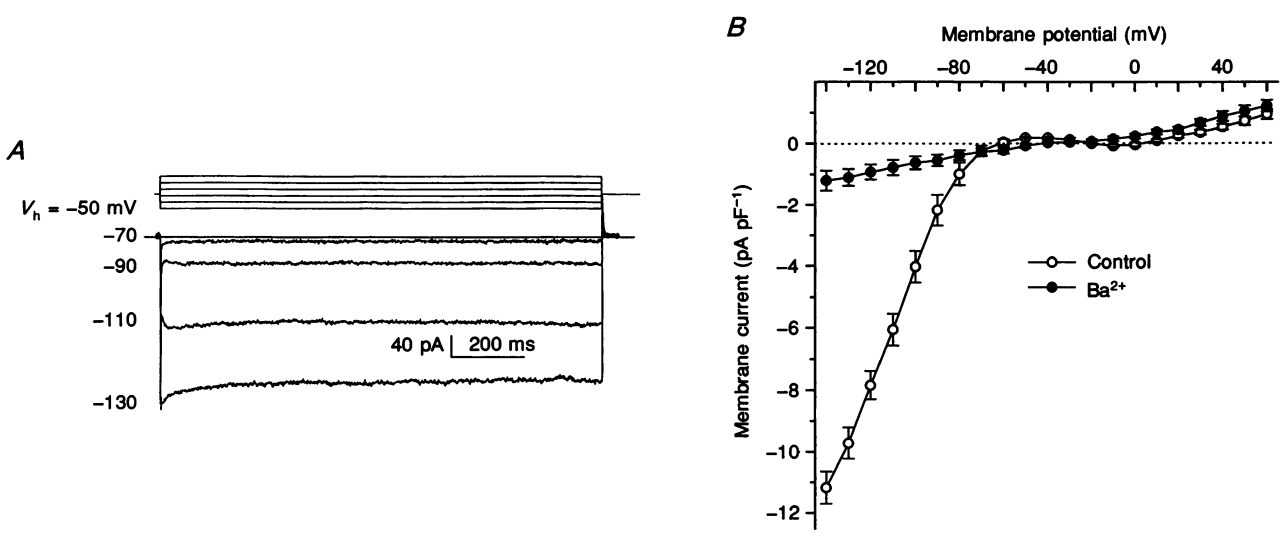

Figure 5. Inward rectifier K^+ currents and block by Ba^{2+}
The immediate inward currents evoked by hyperpolarizations (1000 ms) were large, while very little current was activated during steps to −60 mV and more positive potentials. Block of inward rectifier current by Ba^{2+} (1 mM) was complete and reversible upon wash-out (not shown). Sodium current and T-type Ca^{2+} current were inactivated at the −50 mV holding potential. L-type Ca^{2+} current was blocked by nitrendipine (10 μM). Inward rectifier current–voltage relationship ($n = 6$) is shown in *B*. Cell capacitance, 26 pF.

sensitive component of I_K (i.e. $I_{K,r}$) in neonatal mouse cardiac myocytes. In order to verify the identity of the E-4031-sensitive current as $I_{K,r}$, additional experiments were conducted after the rapid run-down of the slowly activating component of I_K ($I_{K,s}$) had occurred. The E-4031-sensitive difference currents recorded during step depolarizations (1 s) from −40 mV (Fig. 7*A*) demonstrate rectification of the time-dependent current and saturation of the tail current magnitude (Fig. 7*B* and *C*): both features are characteristic of the rapidly activating component of delayed rectifier K^+ current, $I_{K,r}$ described by Sanguinetti & Jurkiewicz (1990). $I_{K,s}$ has been characterized by its very slow activation (seconds) and insensitivity to block by two benzenesulphonamide anti-arrhythmic agents, E-4031 and *d*-sotalol (Sanguinetti & Jurkiewicz, 1990). Our attempts to characterize the pharmacology of $I_{K,s}$ in neonatal mouse cardiac cells were confounded by the extremely rapid run-down of this current.

Sodium current

In spite of the brevity of the action potential, neonatal mouse cardiac myocytes have surprisingly large inward currents that can be fully appreciated only when K^+ currents are blocked. The rapid upstroke of the action potential is consistent with the robust Na^+ currents

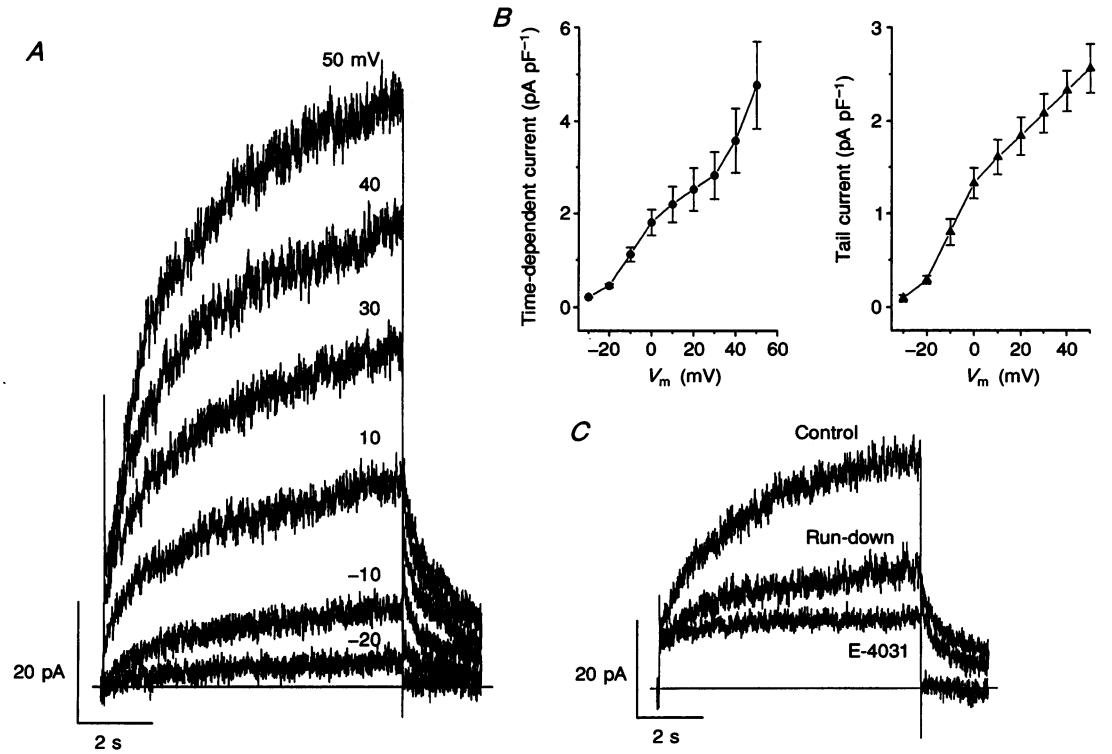

Figure 6. A family of the delayed rectifier K^+ currents (*A*), current–voltage relations for the time-dependent current and the tail current (*B*) and identification of a E-4031 sensitive I_K current (*C*)

A, long depolarizations (8 s) elicited slowly activating time-dependent outward currents. The tail currents evoked upon repolarization to −40 mV (2 s) decayed over time consistent with deactivation of I_K. Currents were recorded in modified Tyrode solution in the presence of 4-AP (2 mM) and Cd^{2+} (500 μM) to block the 4-AP-sensitive I_{to} and the L-type Ca^{2+} current. The depolarized holding potential (−40 mV) steady-state inactivated T-type Ca^{2+} current and I_{Na}. The time-dependent current was defined as the difference in outward current measurements at the beginning and end of the depolarization. Current–voltage relationships ($n = 4$) show that both time-dependent I_K current and peak tail current increase progressively with stronger depolarizations (*B*). Run-down of the time-dependent I_K was rapid (*C*). Soon after impalement, a prominent time-dependent I_K current (control) was recorded during a depolarizing step to +20 mV. Only 4 min later the time-dependent current was dramatically reduced with only a small reduction in I_K tail current (run-down). Wash-in of E-4031 (10 μM) immediately eliminated the tail current and what was left of the time-dependent current (E-4031). This suggests to us that the total I_K current is largely $I_{K,s}$ which runs down rapidly leaving predominantly the E-4031 sensitive $I_{K,r}$. Experiments examining the E-4031 sensitive $I_{K,r}$ current shown in Fig. 7 were performed after rundown of $I_{K,s}$. Cell capacitance, 16 pF.

recorded in these cells. Even with external Na^+ reduced to 40 mM by TEA-Cl replacement, Na^+ currents up to 24 nA in amplitude were recorded (Fig. 8). The currents exhibited gating properties typical of mammalian cardiac myocytes (Lawrence, Yue, Rose & Marban, 1991). The absence of 'abominable notches' in the raw currents, as well as the gradual negative slope region of the current–voltage relation (Fig. 8*B*, $n = 3$), confirms the adequacy of voltage control despite the large magnitude and rapid kinetics of the currents (Cole, 1972). The small size and compact shape of these cells, as well as the absence of T-tubules, all favour uniformity of voltage control.

The sensitivity of the Na^+ current to block by tetrodotoxin (TTX) was examined during cumulative dose–response experiments (Fig. 9). Exposure to TTX in concentrations ranging from 1 nM to 30 μM revealed that the Na^+ channels in neonatal mouse cardiac myocytes bind TTX with low affinity. A power logistic fit (see Methods) of the mean data yielded a half-blocking concentration (IC_{50}) of $2{\cdot}2 \pm 0{\cdot}3$ μM TTX ($n = 4$). An IC_{50} of this order of magnitude is characteristic of the cardiac isoform of the Na^+ channel (Satin *et al.* 1992).

Calcium currents

In addition to a large TTX-resistant Na^+ current, neonatal mouse cardiac myocytes have both T-type and L-type Ca^{2+} currents. The two different populations of Ca^{2+} channels were distinguished by differences in their steady-state inactivation and dihydropyridine sensitivity (Fig. 10). In this experiment, external Na^+ was replaced by *N*-methylglucamine and K^+ was replaced by Cs^+. Depolarizations (180 ms) from −90 mV elicited surprisingly large currents (with 30 mM Ba^{2+} as the charge carrier). When the holding potential was set at −50 mV, a

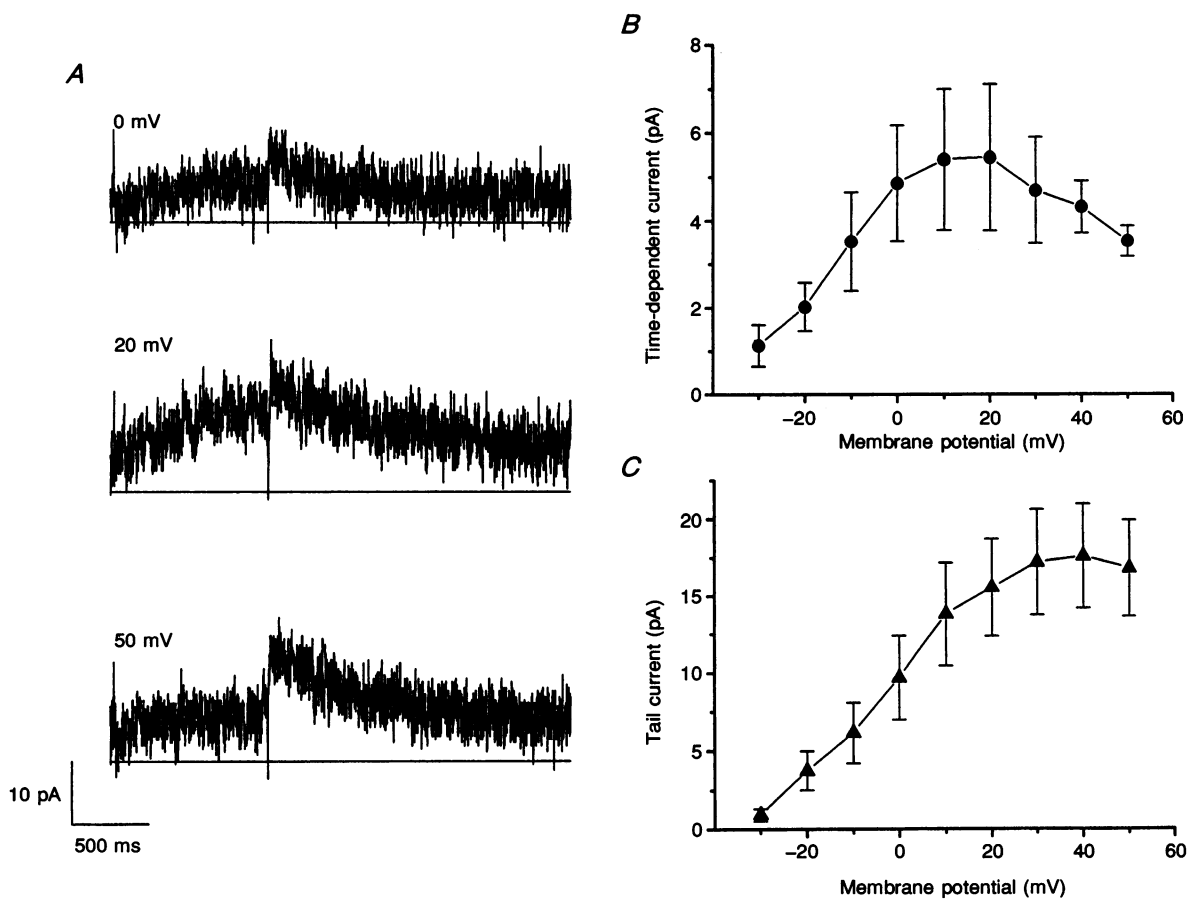

Figure 7. The E-4031-sensitive rapidly activating delayed rectifier K^+ current ($I_{K,r}$) (*A*) and mean current–voltage relationships ($n = 3$) of the time-dependent (*B*) and tail $I_{K,r}$ current (*C*)
Shown are the E-4031 difference currents obtained by subtraction of traces recorded in the presence of E-4031 (10 μM) from the $I_{K,r}$ traces recorded under control conditions (see Fig. 6) after run-down of $I_{K,s}$ during short depolarizations (1 s). Tail currents were completely blocked by E-4031 as was a small time-dependent $I_{K,r}$. The E-4031 sensitive current exhibited rectification of the time-dependent current (above +20 mV; *B*) and saturation of the tail current magnitude (at +30 mV; *C*). The complete sensitivity of the run-down-resistant I_K current to block by E-4031 and E-4031 sensitive current–voltage relationships ($n = 3$) characteristic of $I_{K,r}$ suggest the presence of a small $I_{K,r}$ current in neonatal mouse cardiac myocytes. Cell capacitance, 33 pF.

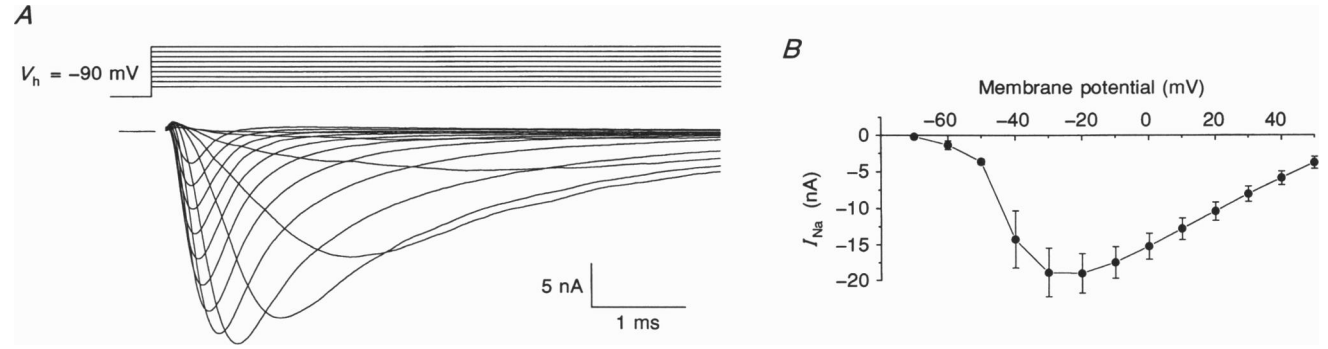

Figure 8. Sodium currents and current–voltage relationship
A, Na^+ currents recorded in 40 mM Na_o^+ measured from 8 to 24 nA. Ni^{2+} (1 mM) was used to block both T-type and L-type Ca^{2+} currents. Cs^+ replacement of K^+ minimized outward currents. The maximum voltage error in this experiment was calculated to be no greater than 5 mV (1 MΩ pipette, 80% series resistance compensation). Cell capacitance, 36 pF. The mean data of 3 such experiments are plotted in *B*. A portion (~100 μs) of the compensation artifact was omitted from the current records for clarity.

portion of the current was inactivated, as expected for T-type channels (Balke *et al.* 1992). Differences in the current records obtained at −30 mV also support the presence of T-type Ca^{2+} channels: the total Ba^{2+} current elicited from −90 mV has an inactivating component, while the current elicited from −50 mV exhibits very little decay during the test pulse.

The relative insensitivity of T-type Ca^{2+} channels to block by the dihydropyridine nitrendipine (Bean, 1985) enabled further separation of T-type and L-type currents. Inactivation of the Ba^{2+} currents elicited from −90 mV in the presence of nitrendipine (10 μM) was nearly complete during depolarization. The inactivation of T-type Ca^{2+} channels is primarily voltage dependent and as such largely unaffected by the substitution of Ba^{2+} for Ca^{2+} as the charge carrier (Bean, 1985). The Ba^{2+} currents elicited from −50 mV were completely abolished by nitrendipine.

Similar experiments were conducted before and during exposure to nitrendipine (10 μM) to assess the contribution of the nitrendipine-insensitive, or T-type current, to the

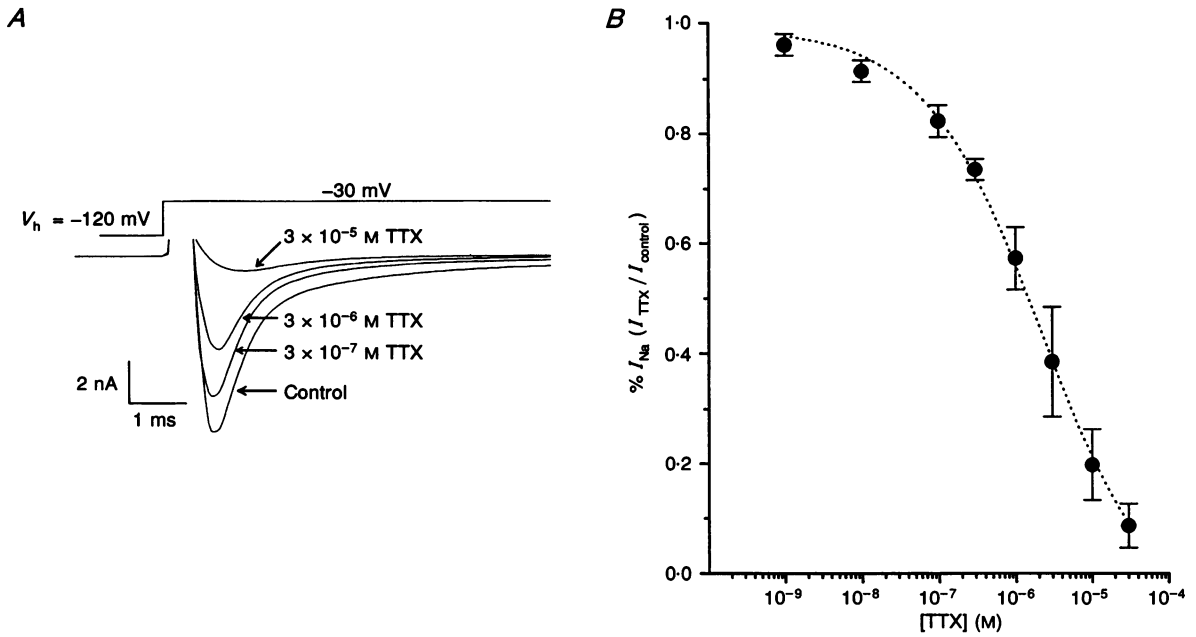

Figure 9. Tetrodotoxin dose–response experiments
Sodium currents were elicited by depolarizations to −30 mV from −120 mV and recorded in the same ionic conditions described in Fig. 8. TTX was applied in cumulative concentrations from 1 nM to 30 μM. A power logistics fit (*B*) to the mean data of 4 experiments, in which I_{Na} recovered on wash-out, indicated an IC_{50} of 2·2 ± 0·3 μM for TTX.

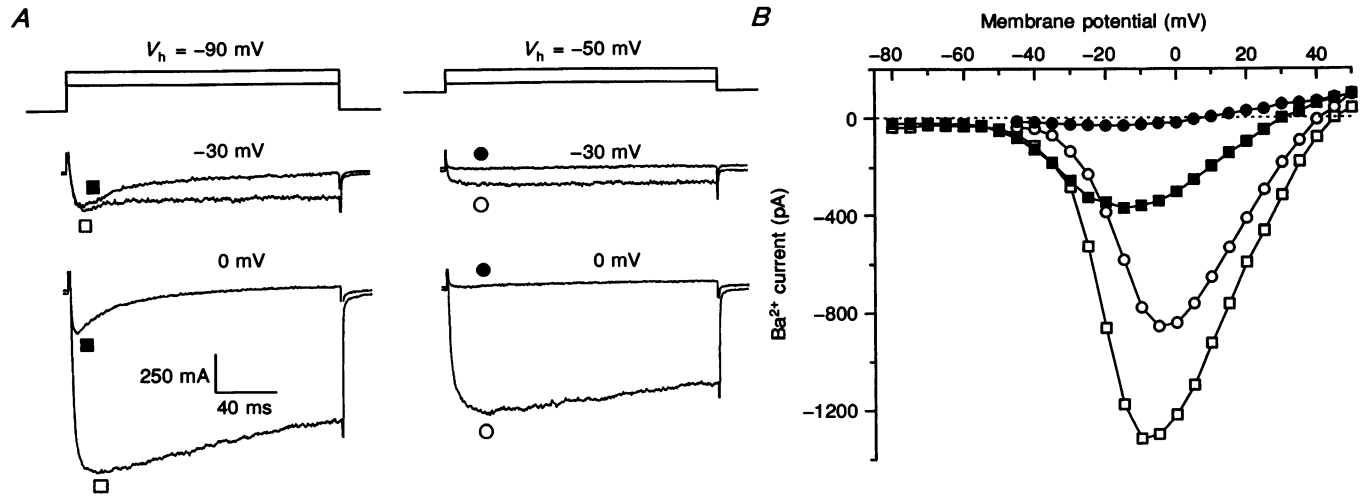

Figure 10. Ba^{2+} currents through T-type and L-type Ca^{2+} channels
Depolarizations (180 ms) elicited Ba^{2+} (30 mM) currents that were larger from −90 mV (□) than from −50 mV (○). Nitrendipine (10 μM) completely blocked the L-type currents from −50 mV (●). The nitrendipine-insensitive current (■) elicited from −90 mV confirms the presence of T-type Ca^{2+} channels. Internal and external recording solutions were Na^+- and K^+-free. The separation of T-type (■) and L-type (○) currents from the total Ba^{2+} current (□) is plotted at each test potential in *B*. Cell capacitance, 25 pF.

total Ba^{2+} current. The pooled data (Fig. 11) indicate that 37% of the total Ba^{2+} current at −10 mV is carried through T-type Ca^{2+} channels in neonatal mouse cardiac myocytes. In other experiments (not shown), Ni^{2+} (1 mM) effectively blocked both T-type and L-type Ca^{2+} currents.

The L-type Ca^{2+} channels in neonatal mouse heart are qualitatively similar to those in adult cardiac myocytes with respect to their activation and inactivation kinetics and their sensitivity to β-adrenergic agonists. The experiment shown in Fig. 12 demonstrates the differences in kinetics between Ca^{2+} currents and Ba^{2+} currents through L-type Ca^{2+} channels. Inactivation is markedly slowed when Ba^{2+} is the charge carrier, such that there is little decay of the Ba^{2+} currents during the depolarizing pulse (200 ms). The differences in inactivation with and without Ca^{2+} are evident by comparing the respective peak current–voltage relations (filled symbols) and the steady-state current–voltage relations (open symbols) shown in Fig. 12*B*. The substitution of Ba^{2+} for Ca^{2+} eliminates Ca^{2+}-dependent inactivation, a characteristic feature of cardiac L-type Ca^{2+} channels (Lee, Marban & Tsien, 1985).

Neonatal mouse cardiac myocytes exhibit the expected increase in L-type Ca^{2+} current in response to β-adrenergic stimulation. Exposure to isoprenaline (1 μM) resulted in roughly an 82% increase ($n = 3$) in the magnitude of the total Ba^{2+} currents elicited at 0 mV (Fig. 13). A quantitatively similar increase in L-type Ca^{2+} current in

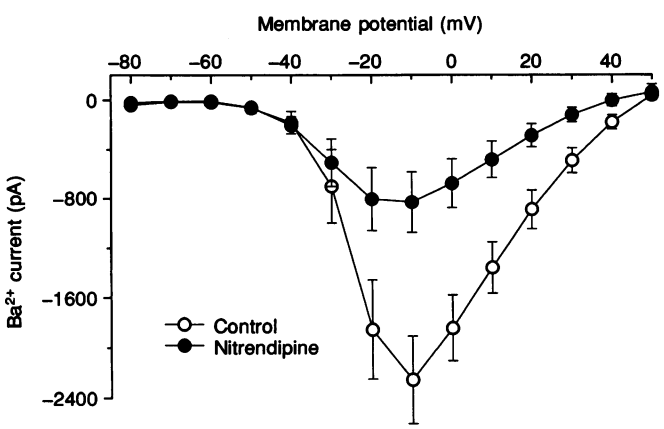

Figure 11. Total and nitrendipine-insensitive Ba^{2+} current–voltage relationships
The means ± S.E.M. are plotted for 4 experiments in which Ba^{2+} currents through Ca^{2+} channels were recorded before and during exposure to nitrendipine (10 μM). The recording conditions were the same as described in Fig. 10.

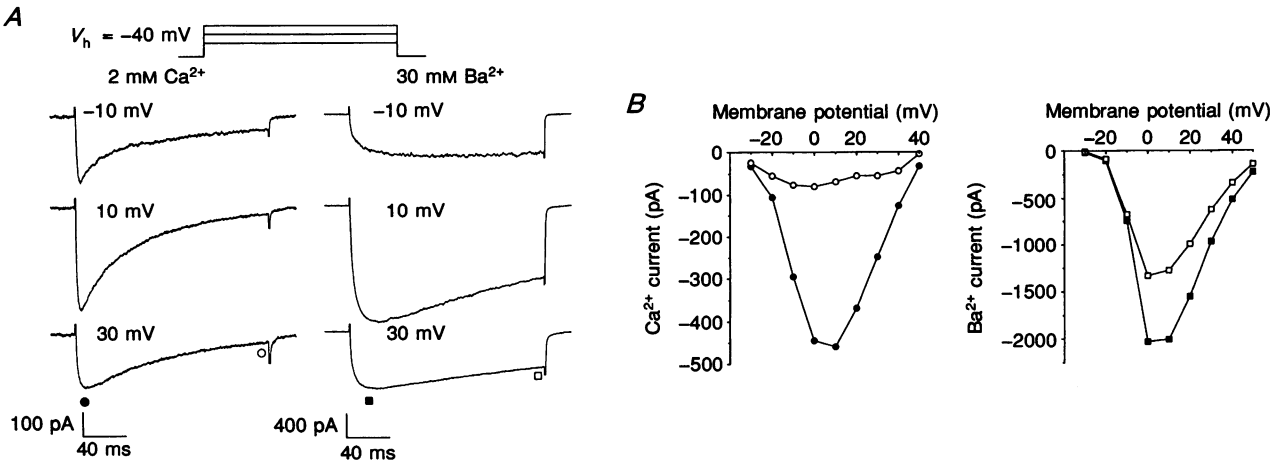

Figure 12. Comparison of Ca^{2+} currents and Ba^{2+} currents through L-type Ca^{2+} channels
Ca^{2+} currents were recorded in a modified Tyrode solution containing 2 mM Ca^{2+}. Ba^{2+} currents were recorded in Na^+- and K^+-free solution containing 30 mM Ba^{2+}. The Ba^{2+} currents (right column of *A*) are roughly 4 times larger and inactivate slower than the Ca^{2+} currents (left column of *A*). The current–voltage relationships (*B*) indicate the peak current (■, ●, peak) and the current remaining prior to repolarization (○, □, steady state). Both recordings were done in the same cell. Cell capacitance, 32 pF.

response to isoprenaline was reported in newborn rabbit (Osaka & Joyner, 1992) and more recently in adult mouse ventricular myocytes (Wolska, Wahler & Solaro, 1993). However, Osaka and Joyner (1992) report that the response in adult rabbit is nearly twice as large as the relative increase in the newborn. Perhaps the β-adrenergic response in mouse is fully developed at birth, while in rabbit the β-adrenergic response reaches its full potential only during postnatal development.

DISCUSSION

The present characterization of transmembrane currents in wild-type neonatal mouse cardiac myocytes provides a basis for future comparative studies of ionic currents in transgenic mouse models. We chose to study the electrophysiology of wild-type murine cardiac myocytes because the mouse has become the prototypical species for transgenic methodology (Grosveld & Kollias, 1992).

Considerations for transgene expression

Transgenic models in which expression is targeted to the heart utilize cardiac-specific promoters to direct the expression of the transgene. The expression of transgenes driven by the ANF promoter, an extensively utilized cardiac-specific promoter (Field, 1988), follow the patterns of regulation of ANF gene expression in the native tissue, which differ between atria and ventricle and in newborn *versus* adult cardiac tissue (Wu, Deschepper & Gardner, 1988). Robust expression of a particular ANF-driven

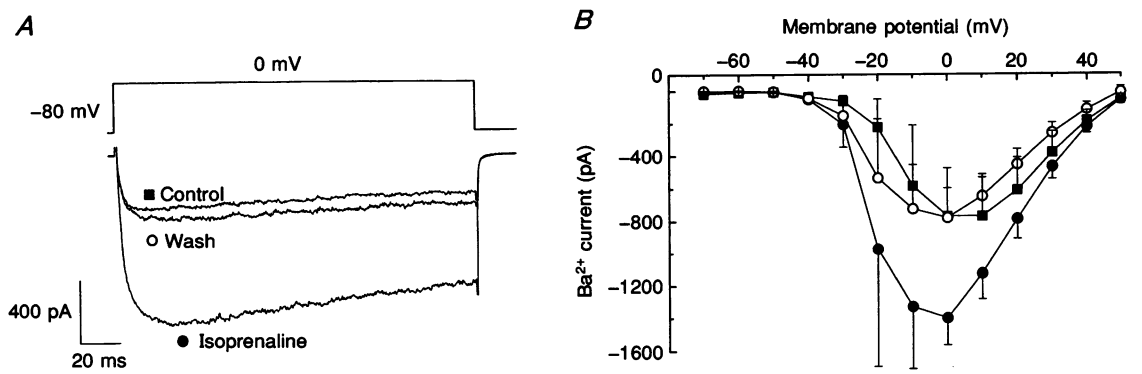

Figure 13. The effect of β-adrenergic stimulation on L-type Ca^{2+} channels
Isoprenaline (1 μM) increased the Ba^{2+} currents elicited by depolarization (180 ms) from −80 mV to 0 mV. The mean data of 3 experiments (*B*), in which an isoprenaline effect was completely reversed upon wash-out, indicate an 82% increase in the total Ba^{2+} current. The recording solutions used are described in Fig. 10.

transgene in the ventricle would be expected to occur only in fetal or neonatal tissue, when ANF gene expression is highest (Wu *et al.* 1988). These considerations prompted us to characterize neonatal, rather than adult, mouse cardiac myocytes.

Physical characteristics

We were immediately impressed with the physical characteristics of neonatal mouse cardiac myocytes in primary culture. It was obvious that these cells, by nature of their small size and compactness, would lend themselves ideally to whole-cell patch clamp studies. The mean cell capacitance in neonatal mouse cardiac myocytes in primary culture was found to be only a fraction of that commonly reported for adult cardiac myocytes (Benndorf *et al.* 1985; Balke *et al.* 1992). Smaller capacitance measurements have been reported for embryonic chick heart cells in culture (7–10 pF) (Aiba & Creazzo, 1993) and freshly dissociated newborn rabbit ventricular cells (16 pF) (Osaka & Joyner, 1992). Such differences in capacitance measurements may be related to species, age, isolation and/or culture procedure and additional to the presence or absence of T-tubules.

The utility of this preparation for studies of excitation–contraction coupling has not yet been investigated. Electron microscopy revealed circular networks of sarcoplasmic reticulum in close association with arrangements of myofibrils. Transverse tubules were not found in any of the electron micrographs ($n = 36$) taken of six cells. However, caveolae were evident in at least one micrograph, and these membranous protrusions could be precursors of T-tubules (Sommer & Johnson, 1968). Although adult mouse heart is known to have T-tubules (Bossen, Sommer & Waugh, 1978), transverse tubules presumably appear only during postnatal development, when the fibre diameter exceeds 7 μm (Bossen *et al.* 1978). The apparent lack of T-tubules is another reason why these cells can be efficiently voltage clamped.

Ionic basis of the action potential

The brevity of the action potential in this preparation is not entirely surprising considering that the heart rate in the mouse exceeds 500 beats min^{-1} (Bossen *et al.* 1978). The action potential duration ($APD_{90} = 42 \pm 2$ ms) recorded in this study for neonatal mouse cardiac myocytes in primary culture is similar to that reported in adult mouse papillary muscle ($APD_{80} = 26{\cdot}0 \pm 2{\cdot}4$ ms; Binah *et al.* 1987) and isolated ventricular myocytes ($APD_{90} = 52{\cdot}4 \pm 3{\cdot}7$ ms; Nilius, Boldt & Benndorf, 1986). Slightly longer action potential durations have been reported for freshly dissociated adult rat ventricular myocytes ($APD_{90} = 44 \pm 1$ ms; Vogel & Terzic, 1989) and in adult rat papillary muscle ($APD_{90} = 47{\cdot}6 \pm 2{\cdot}9$ ms; Binah *et al.* 1987). Curiously, the duration of unstimulated action potentials associated with the spontaneous contractile activity of cultured cardiac myocytes of neonatal rat ($APD_{50} = 197 \pm 22$ ms; Conforti, Tohse & Sperelakis, 1991), embryonic chick ($APD_{90} >$ 200 ms; Mazzanti & DeFelice, 1987) and adult transgenic mouse ($APD_{90} = 96{\cdot}5 \pm 19{\cdot}5$ ms; Steinhelper *et al.* 1990) are much longer.

The reason for the disparity in action potential durations in neonatal mouse and neonatal rat can be understood by contrasting the repolarizing conductances at work. While the predominant repolarizing current in both rat (Josephson *et al.* 1984) and mouse is the transient outward K^+ current, we propose that there are striking differences in the properties of I_{to} in the neonates of both species. If the development of the transient outward current occurs postnatally in the rat (Josephson *et al.* 1984), as it does in the dog (Jeck & Boyden, 1992), then the appearance of I_{to} would explain the age-related shortening of the action potential duration in the rat. We have shown that neonatal mouse ventricle has both the characteristic rapidly inactivating 4-AP-sensitive I_{to} and a very slow or non-inactivating 4-AP insensitive outward current, similar to that described in neonatal dog (Jeck & Boyden, 1992). The rapid activation of both these currents suggests their importance in repolarization and the brevity of the action potential. The similarity in action potential durations between neonatal and adult mouse (Binah *et al.* 1987; Nilius *et al.* 1986) may be explained by a large 'adult-like' I_{to} in the neonate.

The whole-cell transient outward current in adult mouse cardiac myocytes has been reported to be carried by three types of K^+-selective channels (Benndorf, 1988*a*). We speculate that two of these channel types, reported in abundance in adult mouse ventricle (Benndorf, 1988*a*), may preferentially contribute to the 4-AP-sensitive I_{to} and the 4-AP-insensitive outward K^+ current in the neonate. The ensemble average current of the 27 pS channel was reported to inactivate faster and more completely than the ensemble average current constructed from sweeps of 5 pS channel openings (Benndorf, 1988*a*). Alternatively, the 12 pS channel, which was reported to be only one-tenth as abundant in the adult and also had a slowly inactivating ensemble average current (Benndorf, 1988*a*), may contribute to the macroscopic 4-AP-insensitive current in the neonate and be developmentally down-regulated.

In contrast to the size of the transient outward K^+ currents, the delayed rectifier K^+ currents in cultured neonatal mouse ventricular myocytes are very small. Although their contribution to action potential repolarization may be expected to be minimal, clofilium, a delayed rectifier K^+ channel blocker, decreased the beating rate and increased the action potential duration of spontaneously beating cultured neonatal mouse ventricular myocytes (Honore *et al.* 1991). Extremely low densities of delayed rectifier channels have been reported

in embryonic chick ventricular cells, yet they were shown to pass outward current for the duration of the action potential (Mazzanti & DeFelice, 1987). The ability of the delayed rectifier K^+ currents to influence the action potential waveform cannot be ruled out.

A comparison of inward currents

The robust Na^+ current in neonatal mouse cardiac myocytes is the predominant depolarizing current which produces the rapid upstroke of the action potentials shown in Fig. 2. The sensitivity of the Na^+ current in neonatal mouse cardiac myocytes to block by tetrodotoxin is very similar to that reported for adult mouse ventricular myocytes (Benndorf *et al.* 1985). Benndorf (1988*b*) found that a single unitary current level underlies the macroscopic Na^+ current in adult mouse ventricular myocytes. These observations suggest that there is only a single type of Na^+ channel in mouse heart cells, namely the cardiac isoform (Satin *et al.* 1992). In contrast, Na^+ currents in MCM1 cells, a cell line with cardiac-like properties derived from cells isolated from an SV40-induced tumour in the right atrium of transgenic mice, are insensitive to block by tetrodotoxin (100 μM) (Sculptoreanu, Morton, Gartside, Hauschka, Catterall & Scheuer, 1992). The fact that Na^+ channels in cardiac-like cells derived from transgenic mice are different with respect to their sensitivity to tetrodotoxin underscores the importance of characterizing the native currents in wild-type neonatal mouse cardiac myocytes.

The densities of L-type ($I_{Ca,L}$) and T-type ($I_{Ca,T}$) Ca^{2+} currents which we have recorded in neonatal mouse cardiac myocytes are surprisingly large even with Ba^{2+} as the charge carrier. With Ca^{2+} as the charge carrier, the $I_{Ca,L}$ density in neonatal mouse (-10 ± 2 pA pF^{-1}) was large compared with that recorded in embryonic chick cardiac myocytes (Aiba & Creazzo, 1993) and in neonatal rat ventricular myocytes (Rampe & Lacerda, 1991). However, comparable $I_{Ca,L}$ densities have been recorded in newborn rabbit ventricular myocytes ($-13{\cdot}4 \pm 0{\cdot}7$ pA pF^{-1}) under nearly identical conditions with Ca^{2+} as the charge carrier (Osaka & Joyner, 1992). The variety of $I_{Ca,L}$ densities reported in the literature may be related to differences in the pattern of L-type Ca^{2+} channel gene expression in different species. If the expression of L-type Ca^{2+} channels is developmentally regulated, as suggested by several lines of evidence summarized by Sperelakis (1989), then the $I_{Ca,L}$ density may reflect the particular pattern of expression in a given species. The finding that $I_{Ca,L}$ density increases during postnatal development in rabbit ventricular myocytes (Osaka & Joyner, 1991) supports this notion.

We found no evidence for a novel isoform of Ca^{2+} channel ($I_{Ca(fe)}$) in neonatal mouse that has been reported for fetal rat cardiomyocytes (Tohse, Masuda & Sperelakis, 1992). In contrast to $I_{Ca(fe)}$ in fetal rat, the nitrendipine-insensitive Ba^{2+} currents which we recorded from -90 mV in neonatal mouse were completely abolished by changing to a depolarized holding potential (-50 mV) or by the addition of Ni^{2+} (1 mM). Although the peaks of the current–voltage relationships for the nitrendipine-sensitive ($I_{Ca,L}$) and nitrendipine-insensitive ($I_{Ca,T}$) Ca^{2+} currents in neonatal mouse are similar (Figs 10 and 11), the differences in the Ba^{2+} current kinetics, voltage dependence of inactivation and selectivity to block by nitrendipine and Ni^{2+} all argue strongly for two distinct populations of Ca^{2+} channels, namely the classically characterized T-type and L-type Ca^{2+}channels (Bean, 1985).

With the use of isoprenaline we probed the responsiveness of the β-adrenergic system in neonatal mouse. The relative increase in $I_{Ca,L}$ induced by isoprenaline (1 μM) in neonatal mouse cardiac myocytes (82%) was similar to the maximal response in newborn rabbit ventricular myocytes (111%; Osaka & Joyner, 1992). Contrary to the findings of Osaka & Joyner (1992), our inclusion of cAMP (100 μM) in the pipette produced total Ba^{2+} current densities in neonatal mouse cardiac myocytes that were similar in magnitude to those achieved by the addition of isoprenaline to the recording solution ($-61{\cdot}5 \pm 5{\cdot}4$ pA pF^{-1}, $n = 21$ *versus* $-68{\cdot}3 \pm 13{\cdot}4$ pA pF^{-1}, $n = 3$ (from $-37{\cdot}5 \pm 13{\cdot}8$ pA pF^{-1} in the absence of drug, n = 3)). In addition, Osaka & Joyner (1992) found that the maximum response to isoprenaline in adult rabbit ventricular cells was approximately twice as large as in the newborn cells. These differences were not attributed to the postnatal development of the β-adrenergic receptor system but to greater tonic inhibition by G_i, the guanine nucleotide-binding protein which mediates the inhibitory responses, in the newborn (Osaka & Joyner, 1992). Our data suggest that the β-adrenergic receptor system may be fully developed in neonatal mouse cardiac myocytes and thus probably without significant tonic inhibition by G_i.

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