The L type calcium current in single hypertrophied cardiomyocytes isolated from the right ventricle of ferret heart

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Objective: The aim was to study L type calcium current alterations in relation to the action potential lengthening induced by hypertrophy in isolated cardiomyocytes from the right ventricle of ferret. Methods: Chronic pulmonary artery constriction was established in adult male ferrets under anaesthesia. Sham operated animals were used as controls. Four to six weeks later the heart was excised and treated with a mixed collagenase-elastase solution to isolate the right ventricular myocytes. The calcium current was investigated in control and hypertrophied cells with the whole cell configuration of the patch clamp technique. The validity of the model was tested by analysis of the structural and passive electrical characteristics of the cells, which were enzymatically isolated from right ventricles previously overloaded (4 to 6 weeks) by clipping the pulmonary artery. Results: Isolated cells from right ventricles submitted to a chronic pressure overload had well preserved cellular integrity suggesting the absence of myocardial failure. This compensated form of hypertrophy was characterised by a dilated transverse tubular system, which could explain the increased membrane capacity. Such cells developed a prolonged action potential with a less pronounced fast repolarisation phase inducing a higher plateau phase. When studied in physiological Tyrode solution the density and kinetics of the L type calcium current were not apparently modified, but a significant decrease in density was unmasked when sodium and potassium currents were suppressed by external and internal substitution of sodium and potassium by tetraethyl ammonium. Conclusions: The decrease in L type calcium current cannot be involved in the lengthening of action potential observed on hypertrophied myocytes, but it could account for the depressed contractile activity. A noticeable decrease of the transient outward current is suggested to explain the action potential alterations.

The most consistent electrophysiological alteration observed in hypertrophied myocardium is an increase in the transmembrane action potential duration. Such an increase is observed in tissues and in isolated myocytes, whatever the means of chronic overloading used to induce cardiac hypertrophy.

In order to account for this change in electrical properties, the membrane currents responsible for the prolonged plateau and repolarisation phase of the action potential have been investigated. The calcium current (ICa) implicated in excitation-contraction coupling was examined in more detail since hypertrophy also affects the contractile function. From previous experiments on multicellular preparations it has been suggested that the prolongation of the action potential could be attributed to a slower inactivation of ICa. This proposal was strengthened by the increase in the slow time constant of inactivation of ICa observed in myocytes isolated from hypertrophied hearts. However, Ten Eick et al in multicellular preparations and Scamps et al in isolated cells found no difference in the time constants of ICa. Other divergent results have been reported concerning the amplitude and density of the calcium current. Either a decrease in amplitude, or an increase in both amplitude and density, were reported, while an increased amplitude but a constant density has also been shown. These discrepancies could be attributed to various modes of induction of hypertrophy, species differences, or differences in methods of investigation and analysis.

In the present work, we investigated the L type calcium current (ICa-L) of hypertrophied myocytes isolated from the right ventricle of ferret heart submitted to a pressure overload by chronic pulmonary artery constriction. The results showed no alteration in ICa-L density and kinetics when hypertrophied cells were tested under physiological conditions, but a decrease in density was unmasked when the transient outward current was inhibited. Therefore the calcium current alterations cannot explain the characteristic prolongation of the action potential, which would be better accounted for by a decrease in the transient outward current as implied from preliminary results.

Methods

Experimental model
Right ventricular hypertrophy was induced by chronic pulmonary artery constriction in adult male ferrets (Mustella putorius furo) weighing 1200-1500 g, supplied from a controlled breeding stock (Elevage de Beauregard, Roumazières, France). This procedure, initially developed for cats, has been adapted to the young ferret and to the adult male ferret. After premedication with atropine sulphate (Sigma, 0.125 mg) and xylazine (Rompun, Bayer,
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Germany, 5 mg·kg⁻¹), the ferret was anaesthetised (pentobarbital sodium, Sanofi, France, 38 mg·kg⁻¹), intubated, and mechanically ventilated. Under sterile conditions, the left part of the thorax was opened at the fifth intercostal space, a pericardotomy was performed, and the pulmonary artery was dissected free of the aorta. A modified clip (Ligaclip, Ethnor, France) was tightened around the vessel to reduce the luminal area of the pulmonary artery by about 70%. The chest was then closed and the ferret allowed to recover for 4-6 weeks. Sham operated animals underwent similar surgical procedures but without arterial constriction. The experimental animals were handled and maintained in a specific room in our laboratory.

Animal handling and experimentation were approved by the National Animal Ethics Committee.

Preparation of isolated cardiocytes
Isolation of single right ventricular myocytes was performed as previously reported. Briefly, the heart was quickly excised from the chest of the anaesthetised and previously heparinised ferret and rinsed in cold (4°C) Tyrode solution [composition in mM: NaCl 140; KCl 5; CaCl₂ 1.8; NaHCO₃ 4; N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] (HEPES) 20; glucose 11; pH=7.2 adjusted with tris (hydroxymethyl) aminomethane (Tris)]. Since the whole heart had to be kept intact to undergo the digestion procedure, its total weight was taken into account as the first criterion of hypertrophy. It was then cannulated and retrogradely perfused at 37°C for 4 min with a Ca free Tyrode solution containing 20 mM taurine and 0.1 mM ethylene glycol-bis-N,N,N',N'-tetra-acetic acid (EGTA), then for 3 min in this solution without EGTA, and finally for 30-40 min with recirculated enzyme solution containing 60 μM Ca²⁺, 1 mg·ml⁻¹ collagenase, and 0.06 mg·ml⁻¹ elastase (both from Boehringer Mannheim). When the heart was flaccid, the right ventricle was separated, minced in the last solution, but without enzyme, and gently shaken. The isolated cells were filtered, gradually resuspended in a normal Tyrode solution, and kept at room temperature during the electrophysiological experiments.

Experimental procedures and analysis
Transmission electron microscopic observations were performed in order to specify and validate the model by estimating the modifications in the ultrastructure of isolated hypertrophied myocytes relative to normal myocytes. To do this, the isolated cells were fixed for 1 h in a 1% glutaraldehyde solution (0.1 M cacodylate buffer, pH 7.4) and postfixed in 1% OsO₄ cacodylate buffer. The postfixed samples were dehydrated in acetone and embedded in epoxy resin. Ultrathin sections (obtained by means of an ultramicrotome Reicher OMU 2), were stained with uranyl acetate and lead citrate and observed with a Jeol 100C electron microscope.

Figure 1 Transmission electron micrographs (× 12 800) of longitudinal sections of right ventricular myocytes isolated from normal (A) and hypertrophied (B) ferret heart. TT=transverse tubular system; mt=mitochondria; mf=myofibrills; L=lipid droplets.
The passive electrical properties and membrane potential of the cells were also investigated, prior to the I_{ca} studies, as a test of hypertrophy, by means of the whole cell configuration of the patch-clamp technique. The pipettes (1-3 M\(\Omega\)) were filled with a solution containing (in mM): KCl 130; ATPNa\(^{2+}\) 5; MgCl\(_2\) 1; HEPES 10; EGTA 1; pH=7.2 (adjusted with Tris base). The pipettes were connected to the head stage of a patch-clamp amplifier (RK 300, Biologic, Grenoble, France) driven by a microcomputer (PC AX 20, Epson, Nagano, Japan) through an A/D-D/A conversion board (Labmaster TM 40, Scientific Solutions, Solon, USA).

In specific studies of the calcium current, sodium and potassium currents were suppressed by substituting tetraethyl ammonium for both external and internal sodium and potassium ions. Moreover, the intrapipette sodium deficiency suppressed the possible involvement of the current generated by sodium-calcium exchange. Data acquisition and analysis were performed by means of a software package (pClamp, Axon Instruments, Foster City, USA). Data are expressed as mean(SEM).

**Results**

**Cell structure characteristics**

The first evidence of hypertrophy was an increase in the heart weight of operated ferrets (table I), whereas their body weight was similar to that of the sham operated animals. This is expressed by a significant increase of the heart weight/body weight ratio (g.kg\(^{-1}\)) in the group with pulmonary stenosis.

The ultrastructural analysis shows (fig 1) that hypertrophied myocytes display a relatively well preserved intracellular organisation (B) compared to control myocytes (A). However, the hypertrophic state of the myocytes is revealed by an apparent decrease in the number of mitochondria, a widening of the myofibrillar bundles (which could indicate that pressure induced hypertrophy is primarily due to an increase in the cross sectional area of individual myocytes), an enlargement of the transverse tubular system, and an increase in the numbers of glycogen granules. Expanded Z lines were never observed, which confirms that the operated ferrets present a compensated form of hypertrophy without myocardial failure.

**Passive membrane properties and electrical characteristics**

Cell capacitance and series resistance were estimated in whole cell voltage clamp experiments from the capacitive transient elicited by a 10 mV depolarising step from a holding membrane potential of -90 mV. The decay of the capacity transient was fitted to a single exponential function, with a time constant (\(\tau\)) which was increased (\(p<0.05\)) in hypertrophied cells compared to control ones (table II). The membrane capacity (Cm) was also significantly (\(p<0.01\)) increased in hypertrophied cells, as already reported. Expanded transverse tubular system. Thus, from results of Anversa et al., Swynghedauw and Mayoux have estimated that for a 60% hypertrophy the surface area of the T tubule increased by 103% compared to an increase of 35% for the sarcolemma. The uncompensated series resistance measured in these conditions was not significantly changed in hypertrophied myocytes, in accordance with the microscopic observations which have shown a well preserved intracellular integrity.

In hypertrophied cells, the resting membrane potential is slightly depolarised (2.7 mV) compared to control myocytes (table II), in agreement with what has been generally reported (though in some experiments, no significant change has been observed).

**Table I Heart weight to body weight relationships of normal and banded ferrets. Values are means(SEM)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal (n=19)</th>
<th>Banded (n=19)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt (kg)</td>
<td>1.45(0.149)</td>
<td>1.54(0.171)</td>
<td>NS</td>
</tr>
<tr>
<td>Heart wt (g)</td>
<td>9.84(1.81)</td>
<td>11.92(2.05)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Heart wt/body wt (g.kg(^{-1}))</td>
<td>6.85(0.17)</td>
<td>7.84(0.34)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

p values were determined by the Student t test.

**Figure 2 Typical transmembrane action potentials recorded from control (A) and hypertrophied (B) ferret ventricular cardiomyocytes. Note in B the increase of both the plateau amplitude and duration.**

**Table II Summary of the electrical properties of normal and hypertrophied ferret ventricular cardiomyocytes.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal (n=26)</th>
<th>Hypertrophied (n=24)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\tau) (ms)</td>
<td>0.90(0.09)</td>
<td>1.14(0.05)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Cm (pF)</td>
<td>176.7(6.7)</td>
<td>229.1(11.4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Rs (M(\Omega))</td>
<td>5.1(0.4)</td>
<td>5.0(0.4)</td>
<td>NS</td>
</tr>
<tr>
<td>RMP (mV)</td>
<td>-71.8(0.3)</td>
<td>-69.10.4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>APA (mV)</td>
<td>122.8(3.5)</td>
<td>123.1(3.7)</td>
<td>NS</td>
</tr>
<tr>
<td>APD(_{90}) (ms)</td>
<td>480(41)</td>
<td>730(63)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

p values were determined by the Student t test.
A L type calcium current in hypertrophied cardiomyocytes

Hypertrophied

PA

-40

0

20

40

-40

-20

0

20

40

-1.5

-1.0

-0.5

0

-1.5

-1.0

-0.5

0

600 pA

40 ms

-2.0

-1.5

-1.0

-0.5

0

-2.0

-1.5

-1.0

-0.5

0

Figure 3 Comparison of the L type calcium currents elicited by successive depolarisations from 10 normal (filled circles) and 13 hypertrophied (filled triangles) cells tested in normal Tyrode solution. Holding membrane potential (HP)=-40 mV. (A) Superimposition of $I_{Ca,L}$ recorded on a normal cell (filled circles, $C_m=182$ pF) and a hypertrophied cell (filled triangles, $C_m=237$ pF) for depolarisations to $-20$, $0$, $20$ and $40$ mV from the HP. (B) Current-voltage relations of $I_{Ca,L}$ amplitude (a) and density per capacitance unit (b).

Figure 2B shows that the amplitude of the action potential is not significantly changed in hypertrophied cells. However, the duration measured at 90% of repolarisation (APD$_{90}$) is increased by about 50% (table II). This lengthening of the action potential is accompanied by a less pronounced rapid repolarisation phase (fig 2B), so that the plateau potential is maintained at a higher value.

Calcium current analysis

The L type calcium current was first studied in a normal Tyrode solution (containing 1.8 mM Ca$^{2+}$) in the presence of 20 $\mu$M of tetrodotoxin at a holding potential of $-40$ mV in order to get rid of the sodium current. Increasing 200 ms depolarisations (by 10 mV increments) were applied at a frequency of 0.1 Hz and the current amplitude was estimated by the difference between the peak inward current and the current amplitude at the end of depolarising pulses.

Figure 3 illustrates the calcium current/voltage relationships obtained from normal (filled circles) and hypertrophied cells (filled triangles). Both currents display a similar voltage dependence (fig 3B) with an activation threshold around $-30$ mV and a peak amplitude between 0 and $+10$ mV. The current amplitude (fig 3Ba) was larger in hypertrophied cells with a significant increase (p<0.05) for potentials from 0 to $+50$ mV, but the current density (normalised to the respective membrane capacitance reported in table II, fig 3Bb) did not change significantly.

The superimposed current traces reported in fig 3A show that the $I_{Ca,L}$ decay seems faster in control than in hypertrophied cells, an effect which is more evident at
potentials between 0 and 20 mV. Analysis for depolarisation from -10 to +30 mV reveals that the current decay can best be fitted by the sum of two exponentials, the time constants of which are determined by the least squares method. The mean values of the fast ($\tau_f$) and the slow ($\tau_s$) time constants of inactivation, calculated from 12 normal and 10 hypertrophied cells, are expressed as a function of the membrane potential in fig 4A. Both displayed some voltage dependence: $\tau_f$ progressively increased with depolarisations whereas $\tau_s$ described a U shape. Compared to mean values in normal cells (filled circles), $\tau_f$ (fig 4Aa) was slightly smaller in hypertrophied cells (filled triangles) whereas $\tau_s$ (fig 4Ab) was increased, but in both cases the changes were not significant (except for $\tau_s$ at +10 mV; p<0.05).

Figure 5A compares the dependence of calcium channel availability on the potential in the two cell populations. The steady state activation curves (d, fig 5Aa) are determined from the relative membrane conductances calculated according to the method of Isenberg and Klockner. The peak conductance ($g_{Ca}$) is estimated for each potential from the following equation:

$$g_{Ca} = \frac{I_{Ca}}{V - E_{rev}}$$

where $I_{Ca}$ is the peak current for the test potential $V$ and $E_{rev}$ the reversal potential of the calcium current, estimated from the extrapolation of the I/V curve in fig 3B. The steady state activation curve was then obtained by normalising the peak conductance for each test potential. The steady state inactivation curves (f, circles) were obtained with a two pulse protocol. No significant change was observed in the two curve families, which indicates that the voltage range in which the calcium channels are available is not modified by hypertrophy. However, in hypertrophied myocytes at potentials around 0 mV (corresponding to the action potential plateau level), a slight positive shift was generally observed in the inactivation curve, in combination with a corresponding negative shift of the activation curve which could induce an increase in the calcium window current capable of participating in the lengthening of the action potential plateau.

Since calcium currents are studied from a holding potential of -40 mV to avoid contamination by the fast sodium current, the transient outward potassium current is not completely inactivated and the calcium current measurements could therefore be affected by the possible activation of a residual component of the transient outward current. Further experiments were thus performed in which both external and internal sodium and potassium ions were
replaced by tetraethyl ammonium ions at the same molarity. Under these conditions, the steady state activation (fig 5Ba) and inactivation (fig 5Bb) curves were not significantly changed by hypertrophy. But the amplitude of \( I_{\text{Ca,L}} \) (fig 6Ba) was decreased for all depolarisations in hypertrophied cells (open triangles), with significant changes at -10 and 0 mV. This is confirmed in fig 6Bb, in which the calcium current density was significantly decreased for potentials corresponding to the maximum current (ie, from -10 to +20 mV). The superimposition of the current traces (fig 6A) illustrates this decrease of \( I_{\text{Ca,L}} \) in hypertrophied myocytes, and also shows a quite similar decay of the current, which is confirmed by the comparable time constants of inactivation reported in fig 4Bb.

**Discussion**

The right ventricular hypertrophy induced by chronic pulmonary artery constriction is often used as an animal model, even though it does not correspond to a common human pathology. It seems to constitute a relatively pure model of pressure overload hypertrophy, which does not involve significant changes in circulating blood volume, activation of hormonal mechanisms (adrenergic or renin-angiotensin systems), or alterations in the coronary circulation. Although Bishop and Melsen have reported that such an acute pulmonary artery constriction might induce local myocardial necrosis, Cooper et al have shown a similar contractility evolution regardless of whether the constriction is progressive or sudden. In addition, Gwathmey and Morgan have induced a right ventricular hypertrophy on young (70 days old) ferrets by a 60-70% reduction in the pulmonary artery lumen for up to five months without affecting their normal growth and vitality. Table I confirms that the body weight of the banded animals was not significantly different from that of the normal controls.

The increase in the heart weight to body weight ratio in banded animals gives an indication of the degree of hypertrophy of the right ventricle, which could result mainly from an enlargement of the myocytes and an increase in the connective tissue volume. The well preserved intracellular architecture revealed by the microscopic analysis (fig 1) and the unchanged value of the series resistance (table II) both suggest the presence of a compensated hypertrophy stage corresponding to the second stage of hypertrophy proposed by Meerson. At this stage, the increase of the membrane capacity reported in table II could result from the increased area of the surface membrane as a result of the dilatation of the transverse tubular system shown in fig 1B. Microscopic analysis and determination of the passive electrical characteristics of the cells both seem to constitute reliable criteria for evaluating...
the degree of hypertrophy and for validating the present model.

The hypertrophied myocytes isolated in this study develop a prolonged action potential as generally reported whatever the procedure for overloading. The 50% increase in the APD$_{50}$ (table II) is also accompanied by a higher amplitude of the action potential plateau (fig 2B), which increases the action potential duration at any level of repolarisation, as already shown. This indicates that the electrical activity is first affected in its fast repolarisation phase in such a way that the alterations of the action potential configuration could result from any changes in the overlapping active currents flowing during repolarisation.

L type calcium current analysis has shown that the current density is comparable in normal and hypertrophied myocytes when tested in physiological conditions (fig 3), but it is noticeably decreased in external and internal tetraethyl ammonium medium (fig 6), ie, when the transient outward current cannot develop. Therefore the maintenance of $I_{Ca,L}$ amplitude in normal Tyrode solution could result from a concomitant decrease of the overlapping transient outward current which might conceal the actual decrease in the calcium current. This could also explain the apparent slower inactivation of $I_{Ca,L}$ observed under these conditions, because the two time constants measured in tetraethyl ammonium medium are quite similar in control and hypertrophied cells.

**Figure 6**  Current-voltage relations of the L type calcium current from 10 control (open circles) and six hypertrophied (open triangles) cells in both extra- and intracellular tetraethyl ammonium chloride medium. Holding potential (HP)=−50 mV. (A) Superimposed $I_{Ca,L}$ current traces obtained on a control cell (Cm=179 pF) and a hypertrophied cell (Cm=240 pF) for depolarisations to −20, 0, +20, +40 mV from the HP. (B) a=Mean peak $I_{Ca,L}$ amplitude-voltage curves, b=Mean peak $I_{Ca,L}$ density-voltage curves obtained by normalisation of the peak current by the membrane capacitance. The calcium current is significantly decreased in hypertrophied cells at potentials between −10 and +20 mV.
(fig 4B). In this model of overloading, such a decrease of the calcium current has already been suggested. In contrast, Kleiman and Houser have reported an unchanged current density in a similar model, but in their experiments the transient outward current was not completely suppressed by calcium substitution for potassium ions. Moreover, a significant decrease of $I_{Ca,L}$ was recently obtained in ventricular myocytes of cardiomyopathic Syrian hamsters when the transient outward current was suppressed by the presence of both calcium and tetrathyam ammonium in the patch electrode. This decrease of $I_{Ca,L}$ is corroborated by a decrease of 33% in the number of calcium channels, as estimated by [5-methyl]-H nitrendipine binding, in sarcolemma of hypertrophied rat hearts.

The small change in the calcium window current in hypertrophied cells (fig 5) provides the only convenient factor for the involvement of $I_{Ca,L}$ in the maintained depolarisation of the action potential plateau, but it is largely compensated for by the decrease in the current density. Therefore, under our experimental conditions, the calcium current changes cannot explain the increase in the action potential duration, which on the contrary ought to be reduced. The lengthening of the action potential and the associated reduction of the first repolarisation phase could then be better explained by a sharp decrease in the transient outward current, as recently shown in ventricular myocytes from acromegalic rats, an observation which seems confirmed by similar experiments performed in our laboratory. The decrease in the calcium current could then contribute to the depression of contractile activity generally observed in this pathology.

In conclusion, the prolongation of action potential observed in hypertrophied cardiomyocytes cannot result from changes in a single ionic current. It is more likely to result from the sum of several alterations of the different currents which overlap in the potential range of the plateau. In any case, the calcium current decrease cannot account for the action potential lengthening, but could be involved in the depression of contractile activity.

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Key terms: calcium current; right ventricular hypertrophy; isolated myocytes; ferret heart

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