

# Effects of purified perforin and granzyme A from cytotoxic T lymphocytes on guinea pig ventricular myocytes

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**Objective:** Involvement of cytotoxic T lymphocytes (CTL) in heart transplant rejection as well as in viral myocarditis is well established, but the precise mechanisms whereby infiltrating CTL damage the myocardium are unknown. The aim of the study was to investigate how CTL derived perforin, the serine protease granzyme A, and the combination of both, damage guinea pig ventricular myocytes. **Methods:** Action potentials and membrane currents were recorded by means of the whole cell configuration from guinea pig ventricular myocytes. **Results:** Resembling the effects of CTL derived lytic granules, perforin caused gradual myocyte shortening and contracture, leading to complete loss of the rod shaped morphology and to cell destruction. These changes were preceded by shortening of action potential duration and reduction of resting potential and action potential amplitude, followed by complete inexcitability. Granzyme A alone was ineffective, but accelerated the deleterious effects of perforin on the morphological and electrophysiological properties of myocytes. The effects of perforin were further evaluated by measuring membrane currents by means of the whole cell voltage clamp. Perforin induced discrete changes in membrane current, reminiscent of single ion channels, with large conductance and open time of up to several seconds. Linear regression analysis of the channel I-V relations resulted in a conductance of 890 pS and a reversal potential of  $-7.6$  mV. These results suggest that perforin induces large non-selective channels, which can account for most of the observed adverse effects. **Conclusions:** As CTL participate in the immunological rejection of the transplanted heart, it is conceivable, but remains to be shown, that part of this damage is inflicted by perforin containing lytic granules.

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In recent years heart transplantation has become a common surgical procedure for the treatment of protractable heart failure. A major obstacle to this life restoring procedure is the immunological rejection of the transplanted heart induced by infiltrating cytotoxic T lymphocytes (CTL) (both CD4<sup>+</sup> and CD8<sup>+</sup>) in conjunction with antibodies and other factors.<sup>1-4</sup> It has also been suggested that infiltrating CTL contribute to the induction of congestive heart failure in human as well as in murine myocarditis, caused by coxsackievirus B3 (CB3).<sup>5-7</sup> Although the immunology of heart transplant rejection has been thoroughly investigated, the precise mechanism whereby infiltrating lymphocytes damage myocardial function is still unknown.

The mechanisms employed by CTL and natural killer (NK) cells to destroy their targets are the subject of considerable debate, the main issue being the involvement of the pore forming protein, perforin, in cytotoxicity.<sup>8-13</sup> It is commonly thought that upon binding to their target, CTL degranulate and release perforin-containing lytic granules into the intercellular space. In the presence of extracellular Ca<sup>2+</sup>, perforin polymerises into polyperforin, which integrates into the target cell membrane, thereby forming a channel-like pore. These pores are believed to bring about rapid changes in the target cell conductance, possibly leading

to their lysis.<sup>14, 15</sup> In addition to perforin, seven serine proteases, designated granzymes A-G, have been isolated from CTL and NK cells,<sup>16-19</sup> but despite extensive studies their precise role in lymphocytotoxicity is still uncertain. In an attempt to evaluate the role of granzymes in cytotoxicity, we studied the effects of granzyme A alone, and in combination with perforin, on electrophysiological and morphological properties of guinea pig ventricular myocytes. The putative role of granzyme A in lymphocytotoxicity is suggested by its ability to enhance DNA fragmentation in target cells, and by the finding that serine protease inhibitors attenuate the ability of cytotoxic lymphocytes to lyse target cells.<sup>20</sup> Furthermore, several studies have found a good correlation between the expression of perforin and granzyme A, and cytolytic lymphocytes in rheumatoid arthritis.<sup>21</sup>

In a recent study we investigated the effects of a crude lytic granule preparation from CTL on guinea pig ventricular myocytes.<sup>15, 22</sup> Granules caused specific alterations in electrophysiological and morphological properties, and a marked increase in [Ca<sup>2+</sup>]<sub>i</sub> which preceded cell destruction. In the present study we found that comparable effects were induced by perforin, indicating that the cytotoxic effects of lytic granules in guinea pig ventricular myocytes, and possibly during heart transplant rejection, may be attributed at least in part, to perforin.

## Methods

### Preparation of isolated ventricular myocytes

Adult guinea pig (350-400 g) ventricular myocytes were obtained by the enzymatic dissociation procedure.<sup>23</sup> Guinea pigs were anaesthetised intraperitoneally with sodium pentobarbitone, 30 mg·kg<sup>-1</sup>. The chest was opened, the heart rapidly removed, cannulated through the aorta, and perfused with modified Tyrode solution in a Langendorff apparatus. After washing out the blood, the heart was perfused with 50 ml of nominally Ca<sup>2+</sup>-free Tyrode (CFT) solution. Subsequently, CFT solution containing 0.05-0.06% collagenase (type 1, Sigma or United State Biochemical Corporation) was circulated for 25-35 min. Thereafter the heart was perfused with 50 ml of KB (Kraftbrühe) medium.<sup>23</sup> All solutions were oxygenated and warmed to 36±0.2°C. Finally, the heart was removed from the cannula, placed in KB medium, and the suspension filtered through a nylon mesh and stored in KB medium at room temperature (24.0-25.0°C) prior to the experiment. The modified Tyrode solution contained (mmol·litre<sup>-1</sup>): NaCl 140, KCl 4, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub> 1, glucose 10, and HEPES 5. The KB medium contained (mmol·litre<sup>-1</sup>): KCl 70, K<sub>2</sub>HPO<sub>4</sub> 30, MgSO<sub>4</sub> 5, CaCl<sub>2</sub> 0.12, glucose 20, taurine 20, succinic acid 5, pyruvic acid 5, creatine 5, Na<sub>2</sub>ATP 5, and EGTA 0.5. Animal studies were ethically approved by the Technion animal care ethics committee.

### Electrophysiological recordings

After an incubation period (1-2 h) in the KB solution, myocytes were transferred to the recording bath (0.5 ml), mounted on the stage of an inverted microscope (Zeiss IM). The bath was superfused with Tyrode solution at a rate of 1-2 ml·min<sup>-1</sup>. Experiments were carried out at room temperature (24.0-25.0°C). Membrane potentials and currents were measured by means of the whole cell recording technique<sup>24</sup> with the EPC-7 patch clamp amplifier. Electrodes were prepared from glass micropipettes and had a tip resistance of 2-4 Mohm when filled with the pipette solution containing (mmol·litre<sup>-1</sup>): K-aspartate 120, KCl 20, MgCl<sub>2</sub> 3.5, KH<sub>2</sub>HPO<sub>4</sub> 20, Na<sub>2</sub>ATP 3, glucose 10, and EGTA 1. Electrophysiological measurements were carried out during superfusion of the bath with Tyrode solution, after flow was stopped (control measurements), and following application of 5-8 µl perforin in the vicinity of the pipette tip.

### Perforin purification

Perforin was purified from murine CTL (CTLL-R8) following previously published procedures,<sup>14, 25</sup> stored frozen in small aliquots, and thawed once prior to use. The purity of perforin was ascertained by gel electrophoreses, as shown in fig 1. Perforin showed potent Ca<sup>2+</sup> dependent haemolytic activity.

### Preparation of granzyme A

Granzyme A was isolated and purified from lytic granules obtained from two different CTL lines, B6.1 and clone 75, both able to cause specific lysis of target cells expressing H2<sup>d</sup>.<sup>17</sup> The degree of purity was 80%, with 20% contamination by granzyme C, which is enzymatically

inactive. The concentration of granzyme A was approximately 0.180 µg·ml<sup>-1</sup>.

### Statistical analysis

Results are expressed as mean(SEM). To compare means of two populations we used Student *t* test for paired or unpaired observations. Curves relating action potential characteristics versus time were compared by means of the two way ANOVA test.

## Results

### Effects of perforin on the morphology of ventricular myocytes

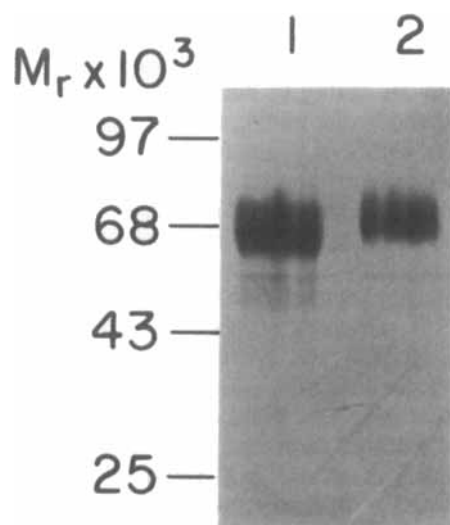
Ventricular myocytes exposed to perforin underwent morphological and electrophysiological alterations, invariably followed by cell destruction. The time course of the cytolytic process was highly variable, with respect to its onset and to the time required for myocyte destruction. Figure 2 depicts changes in myocytes morphology caused by perforin; panels A-D show a myocyte bathed in Tyrode solution (panel A), and at 5, 15, and 20 min after perforin application. It is seen that perforin caused progressive myocyte shortening and contracture, ultimately leading to its destruction. We used two criteria to assess the influence of perforin on morphology (table): T<sub>1</sub>=onset of myocyte shortening as viewed through the inverted microscope at a magnification of ×640; T<sub>2</sub>=myocyte destruction indicated by a complete loss of the rod shaped morphology (fig 1D). At 24.0-25.0°C and in normal Tyrode solution (n=11 myocytes), T<sub>1</sub>=18.8 (SEM 1.6) min and T<sub>2</sub>=37.6(4.5) min.

### Effect of perforin on the action potential; interaction with granzyme A

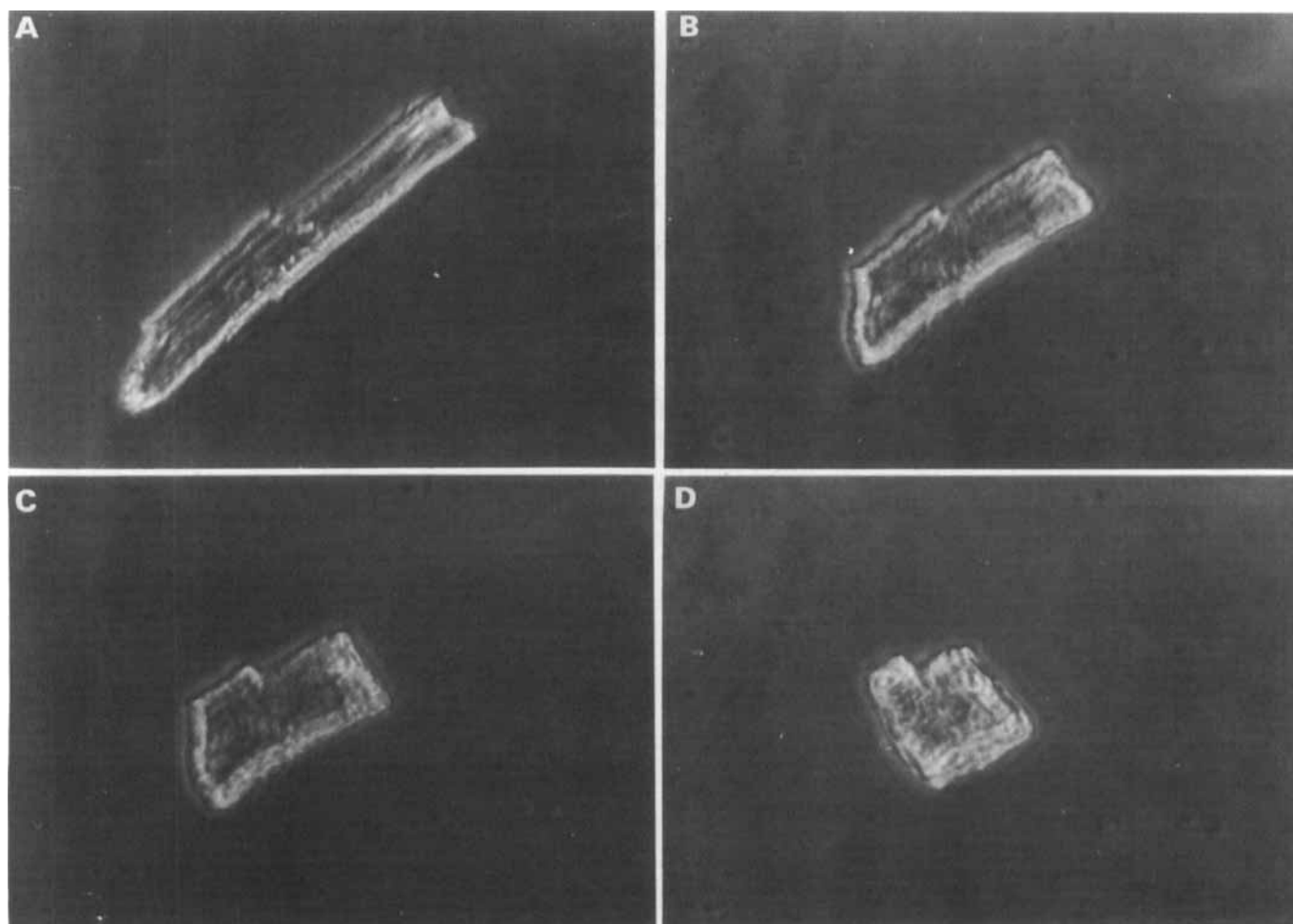
Perforin caused consistent alterations in action potential configuration (fig 3), which if occurring in situ may contribute to the decline in ventricular mechanical function during heart transplant rejection. The first and most typical alteration was a marked shortening of action potential duration, occurring shortly after exposure to perforin and before morphological and other electrophysiological changes were observed. Thereafter, resting potential (V<sub>M</sub>) gradually depolarised and action potential amplitude decreased, followed by electrical inexcitability (not shown). The effects of perforin on action potential characteristics are summarised in fig 4, showing that perforin induced progressive reduction in V<sub>M</sub>, action potential amplitude, and action potential duration at 50% repolarisation (APD<sub>50</sub>). Based on the suspected role of granzymes in lymphocytotoxicity, we tested the effects of granzymes A alone, and in combination with perforin, on myocyte morphological and electrophysiological properties. While granzyme A alone was ineffective, it accelerated (p<0.05) the effects of perforin on action potential characteristics during the first 20-30 min of their combined action (fig 4). Thereafter, the changes in action potential characteristics in the presence and absence of granzyme A were similar. In agreement with these findings, the effect of granzyme A and perforin on myocyte morphology occurred faster than with perforin alone (table).

### Effects of perforin on the membrane current: induction of single channels

We have recently found that the deleterious effects of CTL derived lytic granules were associated with opening of transmembrane channels, with an average conductance of 1390 pS.<sup>15</sup> These channels are probably responsible for non-selective ion fluxes, causing [Ca<sup>2+</sup>]<sub>i</sub> overload and cell destruction. As perforin is considered the major cytolytic



**Figure 1** Perforin was purified from murine CTL (CTLL-R8) on a Sepharose 12 column, analysed on a 10-15% gradient phast gel at 0.1 µg per lane, and stained by silver nitrate. Lane 1 = non reduced; lane 2 = reduced and alkylated. Reduction and alkylation were done with 50 mmol·litre<sup>-1</sup> DDT and 100 mmol·litre<sup>-1</sup> iodoacetamide, respectively.



**Figure 2** Effect of perforin on morphology of a guinea pig ventricular myocyte. Myocyte is shown in the control (A), and at 5, 15, and 20 min (B-D) after application of 8  $\mu$ l of perforin to a Petri dish (3.5 cm) containing myocytes bathed in Tyrode solution (temperature = 24.0-25.0°C). Photographs were obtained by means of an SLR camera attached to an inverted microscope.

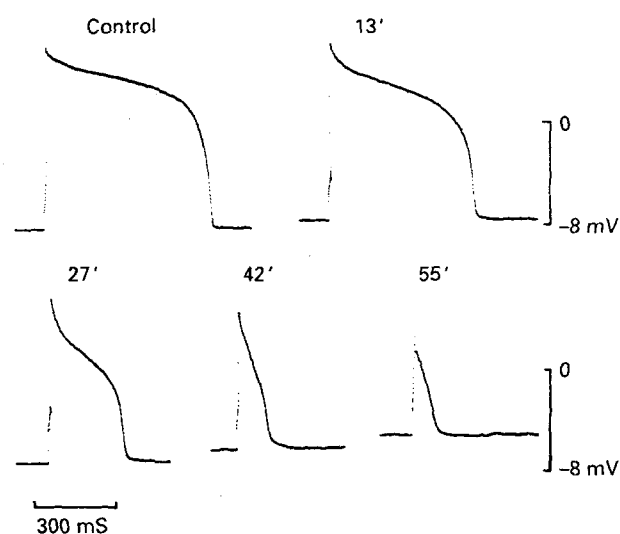
*Morphological changes in guinea pig ventricular myocytes: effects of purified perforin, granzyme A, perforin + granzyme A, and  $[Ca^{2+}]_o$ . Results are means(SEM).*

	$T_1$ (min)	$T_2$ (min)
$[Ca^{2+}]_o = 1.8 \text{ mmol}\cdot\text{litre}^{-1}$ Perforin (n = 11)	18.8(1.6)	37.6(4.5)
$[Ca^{2+}]_o = 1.8 \text{ mmol}\cdot\text{litre}^{-1}$ Perforin + granzyme A (n = 7)	9.5(3.1) <sup>a</sup>	27.6(4.4) <sup>a</sup>
$[Ca^{2+}]_o = 0.1 \text{ mmol}\cdot\text{litre}^{-1}$ Perforin (n = 4)	26.5(2.2) <sup>a</sup>	54.0(4.9) <sup>b</sup>

$T_1$  = beginning of myocyte shortening after addition of perforin;  $T_2$  = complete loss of rod shaped morphology. Morphological changes were monitored through the inverted microscope ( $\times 640$ ). Temperature = 24.0-25.0°C.

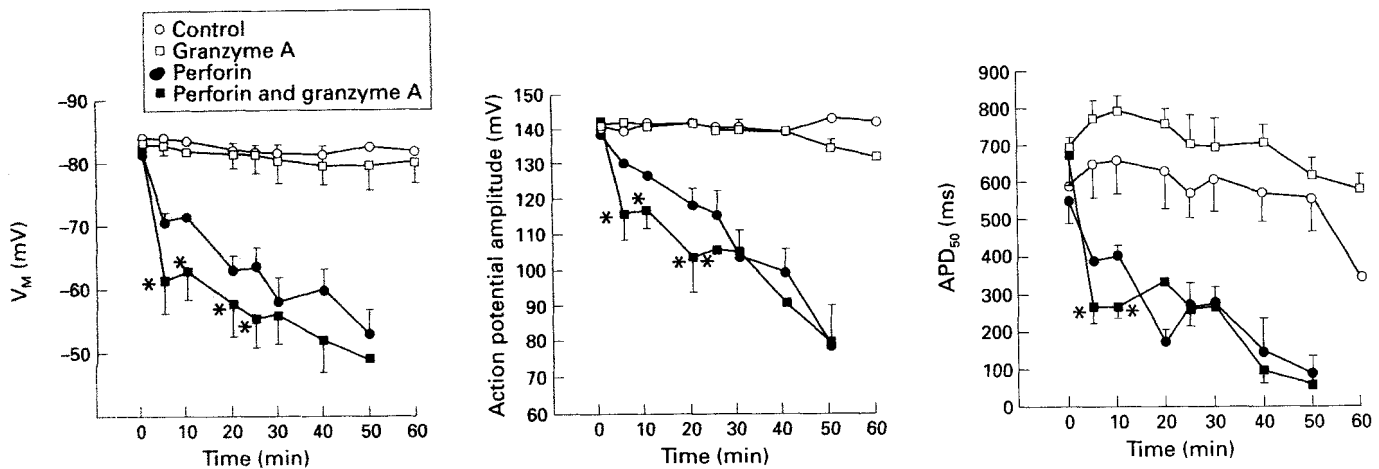
<sup>a</sup> $p < 0.01$ , <sup>b</sup> $p < 0.05$  v control: perforin, 1.8 mmol·litre<sup>-1</sup> Ca<sup>2+</sup>.

component of CTL/NK lytic granules, we tested whether similar channels are induced by purified perforin. Figure 5 depicts recordings of whole cell membrane currents from two different myocytes (panels A and B), showing typical single channels induced by perforin. These channels resemble single channels induced by perforin in EL4 cells<sup>14</sup> and by CTL derived lytic granules in ventricular myocytes.<sup>15</sup> In the experiment shown in panel A, after observing an opening of a perforin channel at a holding potential of -75 mV, the myocyte was clamped to different holding potentials, while continuously recording the membrane

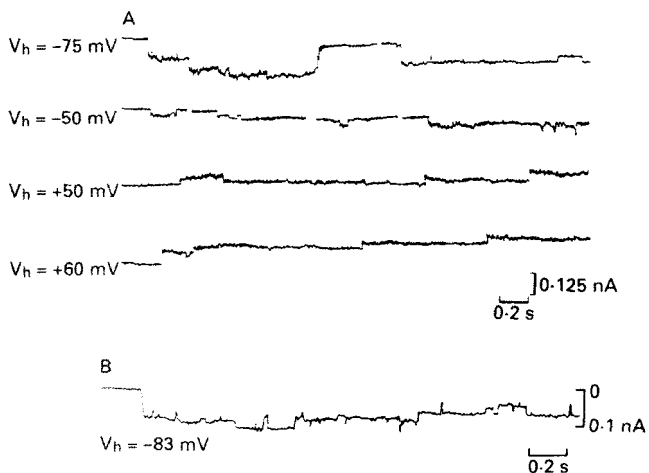


**Figure 3** Influence of perforin on action potential in a guinea pig ventricular myocyte. Action potential is shown in control (Tyrode solution containing 1.8 mmol·litre<sup>-1</sup> Ca<sup>2+</sup>), and at various intervals after application of perforin (8  $\mu$ l into a 0.5 ml recording bath). Temperature = 25.0°C, cycle length = 5 s.

current. This protocol was associated with a decrease in channel current at more depolarised potentials, and a reversal of current polarity at positive membrane potentials. A



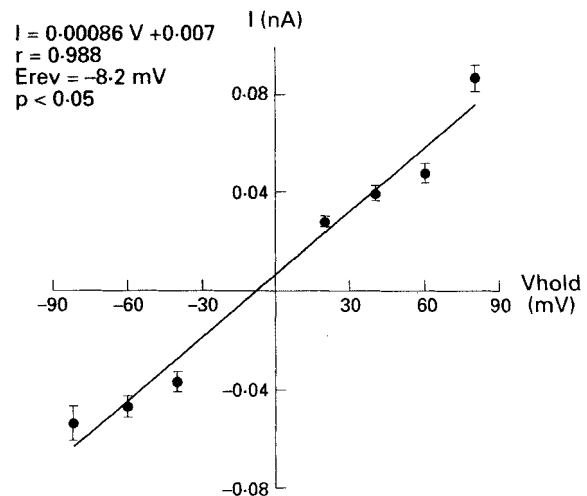
**Figure 4** Effect of perforin, granzyme A, and perforin + granzyme A on action potentials in guinea pig ventricular myocytes. Left: resting membrane potential,  $V_M$ ; Centre: action potential (AP) amplitude; Right: action potential duration at 50% repolarisation ( $APD_{50}$ ). Data are means, bars = SEM. Temperature = 24.0–25.0°C; cycle length = 5 s. \* $p < 0.05$  v control.



**Figure 5** Single channels induced by purified perforin in two guinea pig ventricular myocytes. (A) Recordings of whole cell membrane currents in a myocyte held at different holding potentials. Note reversal of current polarity at positive potentials. (B) A different experiment illustrating the rapid, partial closures in a perforin channel. Temperature = 24.0–25.0°C.

consistent observation in this and other experiments was partial closures of the perforin channel, which are clearly demonstrated in panel B. This interesting phenomenon, which should be further explored, may result from “substates” of the elementary conductance. Thus, although perforin channels differ from common ion channels in their long open time and large conductance, they seem to open and close in a discrete fashion, and therefore are addressed here as single channels.

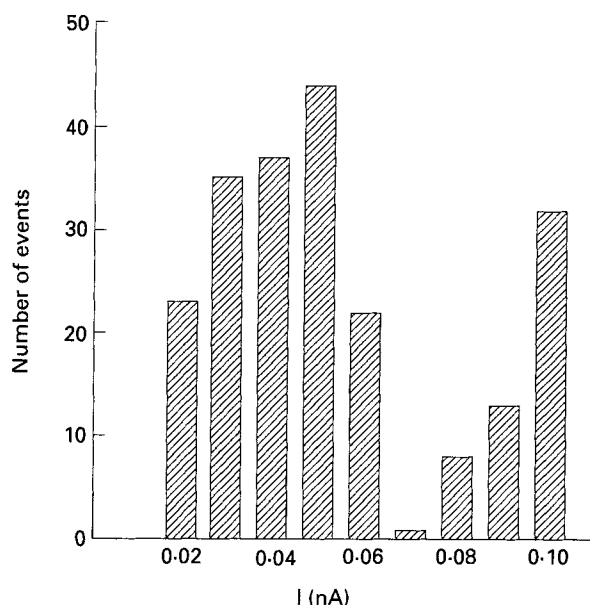
To further characterise the perforin channel, we constructed the current-voltage (I-V) relationships, by measuring the amplitude of the perforin channel at different holding potentials (fig 5A). Current amplitudes used for constructing the I-V relationships were obtained only from distinct channel openings, and not from what appears as “re-openings” after a channel was partially closed (for example, see fig 1A,  $V_h = -50$  mV, and fig 5B). Figure 6 shows I-V relationships constructed in a single myocyte, based on perforin channels recorded at different holding potentials.



**Figure 6** Current-voltage relationships of channels induced by perforin in a guinea pig ventricular myocyte. Each point represents the mean ( $n = 3-15$ ) of the channel current amplitudes measured at a different holding potential. Bars = SEM. The conductance and reversal potential ( $E_{rev}$ ) calculated by means of linear regression analysis are 860 pS and  $-8.2$  mV, respectively ( $p < 0.05$ ). Temperature = 24.0–25.0°C.

The I-V relationships appear linear within the voltage range tested ( $-80$  to  $+80$  mV). Linear regression analysis resulted in a conductance of 860 pS and  $E_{rev}$  of  $-8.2$  mV. In six myocytes in which individual I-V relationships were constructed the conductance and  $E_{rev}$  were 890(21) pS and  $-7.6(1.7)$  mV, respectively. These results suggest that perforin induces large non-selective channels, which can account for most of its deleterious effects.

An important question related to perforin channels is whether they result from a unitary conductance change. This issue is commonly addressed by generating an amplitude histogram with data obtained from a single cell. However, since our routine observations always involved several perforin channels in a myocyte, we generated a histogram containing 214 discrete events pooled from 14 myocytes held at their resting potential [ $V_M = -81.2(0.3)$  mV] (fig 7). Despite the large scatter, it appears that perforin channels are



**Figure 7** Distribution histogram of current amplitudes of perforin induced channels. The histogram includes 214 unitary events recorded from 14 myocytes held at their resting potential of  $-81.2(\text{SEM } 0.3)$  mV. The histogram shows two peaks, at 0.05 nA and at 0.10 nA. Temperature =  $24\text{--}25^\circ\text{C}$ .

distributed in two distinct populations, the first with a peak around 0.05 nA (617 pS), and the second at 0.10 nA (1235 pS), suggesting that perforin may produce two different channels in the target cell membrane.

#### *Ca<sup>2+</sup> ions and perforin induced channels*

Previous studies have shown that typical cytolytic effects of perforin, such as haemolysis of sheep red blood cells,<sup>11</sup> are highly dependent on  $[\text{Ca}^{2+}]_o$ . Theoretically, this can be due to: (1) impairment of perforin polymerisation by low  $[\text{Ca}^{2+}]_o$ , which will be reflected by a delayed onset of channel opening and/or by a smaller single channel current; (2) lowering  $[\text{Ca}^{2+}]_o$  (down to  $0.1 \text{ mmol}\cdot\text{litre}^{-1}$ ), which will not affect channel formation but will delay  $[\text{Ca}^{2+}]_i$  overload through the perforin channels, thereby slowing morphological changes. To test these possibilities we recorded perforin channels in six myocytes exposed to  $[\text{Ca}^{2+}]_o = 0.1 \text{ mmol}\cdot\text{litre}^{-1}$ , instead of  $1.8 \text{ mmol}\cdot\text{litre}^{-1}$  contained in regular Tyrode solution. At  $[\text{Ca}^{2+}]_o = 0.1 \text{ mmol}\cdot\text{litre}^{-1}$ , the time to first channel opening was  $10.7(1.0)$  min, compared to  $10.1(2.3)$  min at  $[\text{Ca}^{2+}]_o = 1.8 \text{ mmol}\cdot\text{litre}^{-1}$ . At  $[\text{Ca}^{2+}]_o = 0.1$  and  $1.8 \text{ mmol}\cdot\text{litre}^{-1}$ , the mean conductances calculated from  $I_{\text{channel}}/V_{\text{hold}}$  (for each experiment) were  $910(83)$  pS and  $890(21)$  pS, respectively. In contrast to that, and in support of the second possibility, the changes in myocyte morphology were significantly delayed by decreasing  $[\text{Ca}^{2+}]_o$  from  $1.8 \text{ mmol}\cdot\text{litre}^{-1}$  to  $0.1 \text{ mmol}\cdot\text{litre}^{-1}$  (table). These results therefore suggest that the changes in  $[\text{Ca}^{2+}]_o$  (in the range studied) modulate the time course of overall perforin cytotoxicity by affecting the rate of  $[\text{Ca}^{2+}]_i$  accumulation and the resulting cell damage, and not by interfering with channel formation and/or conductance.

### Discussion

The objective of the present study was to investigate the effects of CTL derived perforin and granzyme A on the morphology and electrophysiology of guinea pig ventricular

myocytes. While the study was not designed to resolve the controversy concerning the relative contribution of perforin to CTL mediated cytolysis,<sup>8 11-13</sup> we directly tested its activity as a potential candidate mediating the damaging action of perforin containing CTL during heart transplant rejection. We found that perforin induced morphological and electrophysiological alterations in isolated myocytes which, if occurring *in vivo*, may impair myocardial function. Perforin induced action potential shortening, decline in resting potential and action potential amplitude, followed by myocyte contracture and destruction. Since perforin and lytic granules cause a multitude of effects, an important question is whether these can be accounted for by a single mechanism. The mechanism proposed for perforin action is the formation of transmembrane channels (internal diameter of  $150\text{--}200 \text{ \AA}$ )<sup>11 13 26</sup> which are expressed electrophysiologically as discrete changes in membrane conductance. Indeed, a mean conductance of 890 pS and a reversal potential of  $-7.6$  mV found in the present study indicate that perforin induces large non-selective channels in the target cell membrane. These events, which we and others<sup>11 14 15</sup> interpret to result from opening of polyperforin channels, have some features in common with ordinary ion channels. That the conductance changes result from opening of single channels is mainly suggested by (1) the discrete nature of the changes in membrane current (fig 5); (2) the ability of channels to close; (3) the reversal of current polarity at positive potentials; and (4) the histogram of channel current amplitudes, showing two peaks at 0.05 nA and 0.1 nA ( $0.05 \times 2$ ) (fig 7). Two features of this histogram deserve consideration, the large scatter of amplitudes and the presence of two peaks. Similar scatter was reported by Young and his co-workers,<sup>14</sup> and has been accounted for by a large variability in the number of individual perforin molecules forming the pore. The postulated range of 3-20 monomers per pore can certainly explain the large scatter of current amplitudes seen in this and in previous studies. The cause for a double peak histogram is presently unknown, and may result from insertion of two types of perforin channels in the target cell membrane. Channels with properties similar to those reported in this work were induced by granule extracts and purified perforin in planar lipid bilayers,<sup>11 27 28</sup> and in leukaemia EL4 cells [conductance =  $1680(180)$  pS].<sup>14</sup> In those studies, granules and perforin formed large (400-6000 pS), voltage insensitive, ion non-selective channels, permeable to glucosamine, tris(hydroxymethyl)aminomethane ( $\text{Tris}^+$ ), and EGTA, indicating a large functional diameter.

#### *Mechanism of perforin cytotoxicity in ventricular myocytes*

The damage inflicted by perforin can be accounted for by the large perforin channels, which allow non-selective ion fluxes across the cell membrane, resulting in  $[\text{Ca}^{2+}]_i$  overload. We have recently shown that lytic granules cause a dramatic increase in  $[\text{Ca}^{2+}]_i$  (up to  $1 \mu\text{mol}\cdot\text{litre}^{-1}$ ), measured by fura-2 imaging.<sup>22</sup> The reduction in membrane potential seen here and in our previous studies probably results from the loss of  $\text{K}^+$  selectivity and the diminution of chemical gradients. In the study of lytic granules, we explored the mechanisms responsible for APD shortening, and found that granules caused a marked increase in outward currents, accounting, at least in part, for the reduction in APD. As lytic granules and perforin affected action potential similarly, we assume that perforin also shortens APD by a comparable mechanism.

Regarding the changes in morphology, the involvement of  $[\text{Ca}^{2+}]_i$  overload in cell damage is well established.<sup>29</sup> In

addition to inducing several intracellular perturbations (for example, DNA fragmentation,<sup>30</sup> and damage to the mitochondria), raised  $[Ca^{2+}]_i$  can directly affect cytoskeletal organisation and thus induce surface protrusions known as blebs.<sup>31</sup> These alterations could result from indirect interactions between  $Ca^{2+}$  ions and various structural components of the cytoskeleton. For example, increased  $[Ca^{2+}]_i$  can activate proteases that cleave actin binding proteins, eliminating the plasma membrane anchor for the cytoskeleton.<sup>32, 33</sup>

#### Contribution of granzyme A to perforin action

In the present study we showed that while granzyme A alone is ineffective, it accelerates the deleterious effects of perforin. This observation adds to previous observations suggesting that serine proteases play an important role in cell mediated cytotoxicity (reviewed by Jenne and Tschopp<sup>18</sup>). For example, it has been shown that granzyme A enhances DNA fragmentation in target cells, and serine protease inhibitors decrease the ability of CTL to lyse their targets.<sup>20</sup> Besides from potentiating perforin action, granzyme A may be responsible for the differences in the properties of channels induced by perforin and lytic granules; the calculated conductance of the perforin channel is 890 pS, while that of granules is 1390 pS. Perhaps the presence of granzyme A in lytic granules facilitates perforin polymerisation, thereby producing larger channels with higher conductance. The precise mechanism responsible for the interaction between perforin and granzyme A has not been studied in the present work.

#### Estimates of perforin channel dimensions

A simple estimate of single channel conductance ( $G_{ch}$ , see Hille<sup>34</sup>), based on a cylindrical channel model of length "z" (70 Å), radius "r", and  $R_s$  = the volume resistivity of the electrolyte ( $1.3 \times 10^{10}$  ohm-A), is given by:

$$G_{ch} = (\pi r^2 / R_s) / (z + (\pi r / 2))$$

Using the calculated conductance of 890 pS (fig 6), and the low and high  $R_s$  values, the estimated channel diameters were 33 Å and 57 Å, respectively, compared with lytic granule channel diameters of 42-85 Å.<sup>15</sup> In agreement with the large conductance and the non-selectivity of the perforin channel, our estimates of channel diameter (34-57 Å) greatly exceed the predicted sizes of  $Na^+$  and  $K^+$  channels (3-5 Å).<sup>34</sup> On the other hand, they are consistent with the small-type (polyperforin 2) tubular lesions of 50 Å diameter induced by cloned cytolytic cell lines, but are smaller than the large lesion of 160 Å induced by polyperforin 1.<sup>11, 35, 36</sup> Our estimates fall short of the 100 Å pores calculated from flux rates of a single perforin pore in human erythrocyte ghosts by means of fluorescence microphotolysis.<sup>37</sup> In conclusion, the present study suggests a potential mechanism for the damage induced by perforin-containing CTL to myocardial tissue during heart transplant rejection. As killer lymphocytes participate in the immunological rejection of transplants, including the heart, it is conceivable that part of this damage is inflicted by secreted perforin-containing lytic granules. To verify this theory we are currently investigating the electrophysiology of ventricular myocytes subjected to direct attack by CTL rather than by isolated cytotoxic granules.

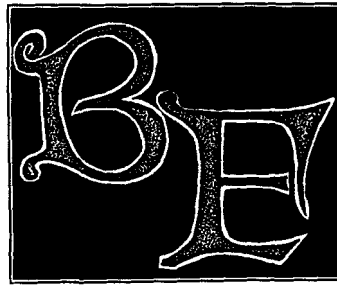
Key terms: cytotoxic T lymphocytes; purified perforin; transplantation graft rejection; guinea pig ventricular myocytes.

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