Results and discussion. Arterial rings show prostacyclin-like activity, as demonstrated by inhibition of platelet aggregation. Within 30 min of removal, there is only a slight but not significant decrease in the availability of prostacyclin. However, immediately after removing, the number of endothelial cells shows a continuous decrease in light and scanning microscopy. After 10 min, the number of endothelial cells falls beyond 10% of the starting value. After 15 min, endothelial cells almost completely disappear. As the storing buffer is changed every 3 min, it can be concluded that the nearly complete disappearance of endothelial cells does not alter significantly the availability of prostacyclin-like substance.

Incubation with Ketoprofen, a prostaglandin synthetase inhibitor, prevents prostacyclin production; boiling for 30 sec destroys the platelet active compound as well as incubation of tissue in buffer for 10 min at 37 °C.

Morphological control of Moncada's bioassay[1,2,9,10] for prostacyclin measurement shows that after desquamation of endothelial cells the prostacyclin activity does not alter significantly. This recalls some evidence, as found by Hornstra[11] in a different experiment, that some other than endothelial cells in the subendothelial part of the intima have a similar ability to produce prostacyclin. Though there is an activity in the media[11,12], this is not comparable with the quantity found in the subendothelium. Probably this activity is due to some special differentiated cells[13,14] found in the intima[15]. This view is also supported by the fact that a thrombus during organization has an activity[16], probably derived from the so-called pseudoendothelium[17]. In contrast, removal of endothelium causes an immediate formation of a mural platelet thrombus in vivo[18] and in vitro with rabbit[19] and human[20] arteries. Rubbing of endothelium with sand paper[11] increases prostacyclin activity, whereas mincing has no effect on the renal prostacyclin production[21]. Since it can be accepted that, over an intact endothelium mural platelet, thrombus cannot be found[22], the theory cannot completely be upheld that platelet thrombus is formed after endothelial desquamation, because there is not enough prostacyclin production to prevent the genesis of a mural platelet thrombus.

Beside this fact, we suggest that not only the activity in normal cells[1,12] and tissue[1,21,23-25], but especially the basal[1,26] activity and availability[24,26,27] of altered or transformed cells[13,15], or metabolically influenced tissue, namely in uremia[24,27] myocardial infarction[28] etc, demonstrates the very important, yet uncleared role of prostacyclin in atherosclerosis.

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Chemically mediated cell-to-cell contractile activation in isolated frog atrial cardiac cells[1]

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Summary. A dying single frog atrial cardiac cell liberates an unknown substance which diffuses away from the dying cell and activates contractile activity in other isolated intact single cardiac cells within the vicinity of the dying cell.

Recently we described the successful preparation of isolated intact single frog atrial cells by dispersion of frog atrial tissue with a combination of trypsin and collagenase[3]. We had hoped that it would be possible to record intracellular potentials from these single cells with microelectrodes, but we found that the single cell dies immediately upon penetration of its membrane with a microelectrode. While attempting these microelectrode recordings, we observed the curious phenomenon that neighboring cells not in any obvious direct contact with the impaled cell often gave twitch-like contractions after the death of the impaled cell. The time interval between when a cell was impaled and a neighboring cell contracted increased as the distance between the cells increased suggesting that a substance was

released from the impaled cell which diffused away from the dying cell and activated contractile activity in neighboring cells when it came in contact with them. The purpose of this paper is to present data which strongly suggests that the dying cardiac cell liberates some substance which is capable of activating contraction in intact cardiac cells.

Materials and methods. Isolated frog (*Rana catesbeiana*) atrial cells were prepared by trypsin-collagenase digestion of intact atrial tissue as described previously[3]. Cells suspended in Ringer solution (NaCl = 111 mM, KCl = 5.4 mM, $CaCl_2$ = 1.8 mM) were added to tissue culture dishes (Falcon tissue culture dish No. 3001) and allowed to settle and attach to the surface of the culture dish. To aid cell attachment to the culture dish surface, poly-l-lysine (mol.wt ≃ 70,000) was attached to the culture dish surface by adding a few drops of a poly-l-lysine solution (1 mg/ml) to each culture dish for a few minutes followed by a thorough rinsing of the culture dish surface with deionized water to remove excess poly-l-lysine[4]. An unfilled microelectrode was used as a microprobe for impalement of a cell. A closed circuit TV-video tape-microscope system was used to both observe the cells and record the data for analysis of the time interval between cell impalement and neighboring cell(s) contraction(s) as well as for determination of the distance between the impaled cell and the neighboring cell(s). In most experiments the number of cells within a 400 µm radius of the impaled cell was generally so few as to preclude the analysis of multiple cell responses to a single cell impalement.

Results. Microprobe impalement of an isolated frog cardiac cell invariably produced immediate cell death. Surprisingly, neighboring cells not in any obvious direct contact with the impaled cell often gave twitch contractions after the death of the impaled cell; these twitch contractions appeared to be similar in duration and magnitude to those obtained by direct electrical stimulation of the isolated cells. A plot of the time interval of cell impalement-neighboring cell contraction versus the cell separation distance (intercellular distance) is given in figure 1. The closed-circles give the time-distance relationship in the absence of fluid flow between the cells; the 500 data points used in this analysis were obtained from impalements of 464 cells. In this case it is apparent that the slope of the time-distance relationship increases as the intercellular distance increases as would be expected if a chemical were diffusing away from the impaled cell and activating neighboring cells when it came in contact with them. This figure also demonstrates that cell-to-cell contractile activation can occur at intercellular distances greater than 400 µm.

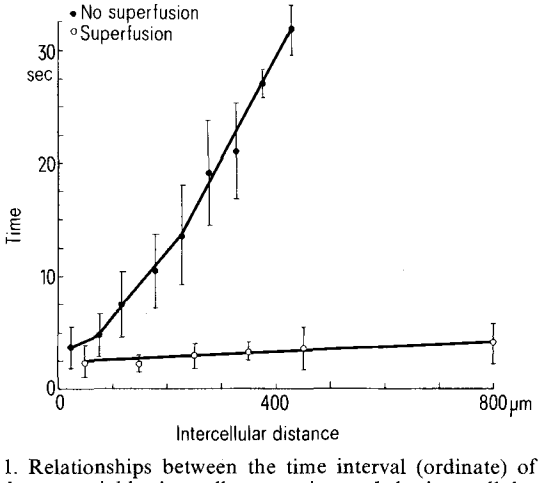

Fig. 1. Relationships between the time interval (ordinate) of cell impalement-neighboring cell contraction and the intercellular distance (abscissa). The closed circles give the relationship in the absence of fluid flow (no superfusion) between the cells; the open circles give the relationship in the presence of fluid flow (superfusion). The data points (500 for no superfusion, 63 with superfusion) were grouped within distance intervals (50 µm for no superfusion, 100 µm for superfusion) and the mean time ±1 SD are plotted as a function of the mean distance within the distance interval; the mean time data plotted at 800 µm with superfusion included data points within a distance interval of 700–900 µm.

To determine if structures not visible with the light microscope interconnected the cells in the culture dish, cells were plated onto poly-l-lysine coated glass cover slips and viewed with the scanning election microscope. The scanning electron microscope revealed the existence of filaments ranging in size from 600 Å to 0.2 µm diameter which interconnected some of shown cardiac cells. Examples of these filaments are shown in figures 2 and 3. Figure 2 is a relatively low power scanning electron micrograph showing a filament (arrow) interconnecting 3 cardiac cells plus some cellular debris. Figure 3 shows 3 filaments running over the top of a single cardiac cell and connecting to cellular debris. The inset in the upper left of figure 3 shows the 2 filaments at the right of the cardiac cell at high magnification to demonstrate the attachment of the filaments to the surface of the cardiac cell.

Several different experiments were performed to rule out the possibility that some signal (e.g., electrical or mechanical) was being communicated from the impaled cells to its neighbors via the filamentous structures thereby causing contractile activation of the neighboring cell. First, it was found that local electrical stimulation which elicited contractile activation of a single cell did not lead to subsequent contractile activation of neighboring cells as might be expected if the cell-to-cell contractile activation were by an electrical stimulus (e.g., an action potential) or mechanical stimuls (e.g., force applied to the filaments) mediated through the thin filaments interconnecting the cells. Second, contractile activation of a cell attached to a poly-l-lysine coated glass probe could be accomplished when a cell on the surface of the dish was impaled while in close proximity to the cell on the glass probe. The cell on the glass probe could then be moved to a new region of the culture dish and the experiment repeated successfully. In

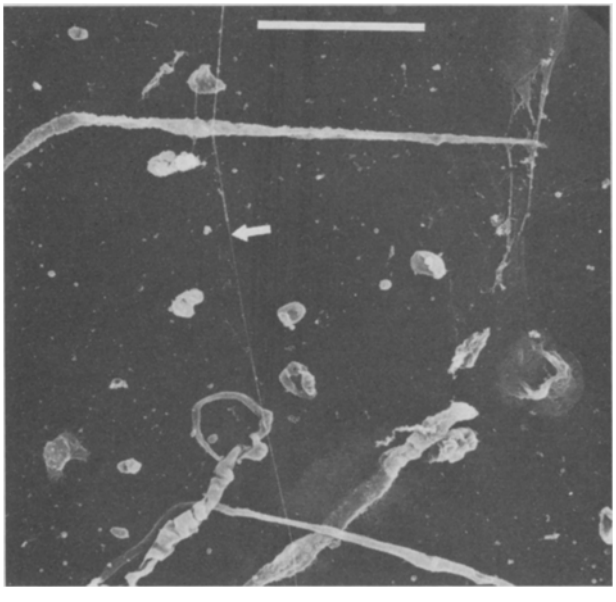

Fig. 2. Low power scanning electron micrograph of 3 isolated single cardiac cells interconnected by a filament (arrow). Calibration bar represents a length of 50 µm.

these experiments the cell on the glass probe was in the vicinity of the cell on the dish surface for only a very brief time (less than 30 sec) prior to impalement of the cell. Thus, if the filaments played a crucial role in the cell-to-cell contractile activation, the filaments must have formed rapidly and broken easily upon translation of the cell through the fluid in the culture dish. Third, the time interval between cell impalement and neighboring cell contractile activation was dramatically reduced when the cells in the culture dish were superfused provided the neighboring cell was 'downstream' to the impaled cell. The results of this experiment are also presented in figure 1 (open circles). In the presence of fluid flow between the impaled cell and the neighboring cell, the time-distance relationship appeared to be fairly linear and neighboring cell contractile activation could be accomplished rapidly (less than 5 sec) over relatively great distances (800 µm); the 64 data points obtained in these experiments were obtained from impalements of 54 cells. The decrease in the time-distance relationship associated with superfusion is consistent with an enhanced speed of transport of a chemical substance by the fluid stream. A decrease in the time-distance relationship with superfusion would not be expected if the cell-to-cell contractile activation were by an electrical or mechanical stimulus transmitted through the thin filaments interconnecting the cells.

Discussion. The data presented in this paper demonstrate that the dying cardiac cell liberates a substance which activates contractile activity in neighboring cells when it comes in contact with them. At present the composition of this substance is unknown.

Qualitative estimates as to the size of the substance and its effective concentration can be made if it is assumed that the substance diffuses away from the impaled cell as from an instantaneous point source located at the center of the base of a hemisphere. In this case, a monophasic concentration wave of the substance would propagate away from the impaled cell. The peak amplitude of the concentration wave would decrease with distance from the impaled cell according to the equation $C_{max} = 0.15\ Q/r^3$, where Q is the amount of the substance released from the impaled cell and r is the distance from the impaled cell[5]. The equation $6\ DT = r^2$ gives the relationship between the diffusion coefficient (D) of the substance and the time (T) of arrival of the peak of the concentration wave as a function of distance (r) from the point source[5].

The volume contained within a single frog atrial cell is on the order of 5–10 pl. If the intracellular concentration of the substance were 100 mM (a value undoubtedly too high) and the total amount of the substance were released instantaneously upon cell impalement, then the magnitude of Q would be on the order of 0.5–1.0 pmole. In this case, the peak concentration of the substance 400 µm from the point source would be on the order of 1–2 µM. Thus, it seems reasonable to conclude that the substance must be effective in relatively small concentrations (µmolar or less). This relatively small effective concentration would tend to rule out potassium as a candidate for the substance, since activation of contraction by potassium requires an extracellular potassium concentration in excess of 30 mM. Furthermore, the cell-to-cell contractile activation still occurred when the cells were exposed to an extracellular potassium concentration of 28 mM.

If it is assumed that contractile activation of the cell occurred when the peak of the concentration wave of the substance reached the cell, then a plot of the time interval between cell impalement-neighboring cell contraction versus the square of the distance between the cells should give a straight line relationship from which the diffusion coefficient can be calculated ($D = r^2/6T$). A time-distance squared analysis of 392 data points did give a straight line relationship having a correlation coefficient of 0.81, a slope of 1.9×10^4 sec/cm^2, and a time intercept at zero distance of 4 sec. From the slope of this line, a diffusion coefficient of 8.8×10^{-6} cm^2/sec can be calculated. This diffusion coefficient suggests that the unknown substance has a relatively small mol. wt; the diffusion coefficients of substances having mol. wt in the range of 60–300 have diffusion coefficients in the range of 13×10^{-6} to 7×10^{-6} cm^2/sec[6]. Acetylcholine, for example, has a diffusion coefficient of about 8×10^{-6} cm^2/sec.[5]

The identity of the contractile activating substance and the mechanism by which it activates contraction in intact cardiac cells remains to be determined. Recently, McClellan et al.[7] reported the presence of a cardiotonic substance in homogenates of rat ventricle. When 'chemically skinned' rat ventricular tissue was exposed to this substance, significant tension was developed by the tissue even when pCa was 9.0. It was not reported whether or not this substance was capable of activating contraction when applied externally to cardiac tissue having intact cell membranes. However, the substance was active at relatively small concentrations, since activity was retained when the homogenate was diluted 2500-fold.

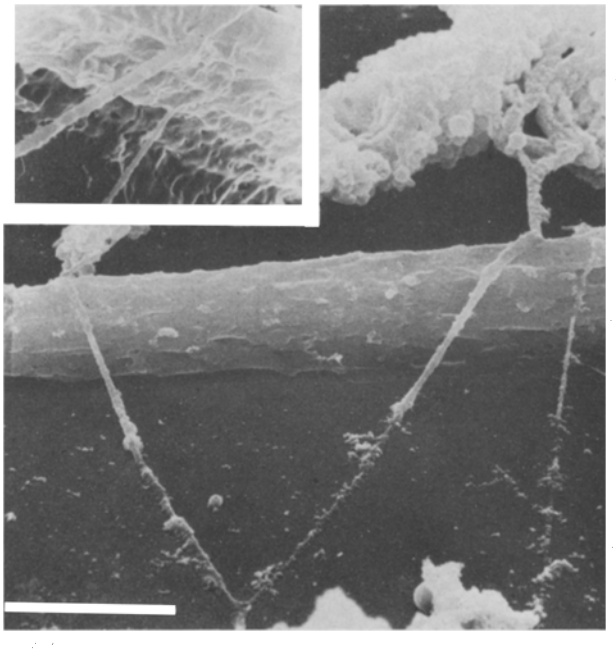

Fig. 3. Scanning electron micrograph showing 3 filaments overlying a single cardiac cell. The inset (upper left corner) shows the 2 filaments at the right of the cardiac cell; the preparation was rotated to show the attachment of the filaments to the surface of the cardiac cell. The calibration bar represents a length of 5 µm for the micrograph and 3.1 µm for the inset.

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2 We wish to thank Dr Alan Chapman of the Department of Anatomy for doing the scanning electron microscopy of the cardiac cells.
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