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Contraction and Volume Changes of Glycerol Treated Rabbit Polymorphonuclear Leukocytes Induced by ATP and Ca^{2+} [1] (38124)

LONG S. HSU AND ELMER L. BECKER
(Introduced by I. H. Lepow)

The Department of Pathology, University of Connecticut Health Center, Farmington, Connecticut 06032

Hoffman-Berling proposed that cells move due to the contraction of actomyosin-like proteins contained in them. This hypothesis was originally based on the observations that exogenous ATP and Mg^{2+} are able to induce a microscopically observable contraction of glycerinated fibroblasts, sarcoma cells and amoebae and that this phenomenon shares many common features with the contraction of glycerinated muscle fibers by ATP and Mg^{2+} as first observed by Szent-Györgyi (1). The hypothesis has received considerable support based on the findings that actomyosin-like proteins can be isolated from many cells varying from amoebae to animal cells (2–8) and actin-like filaments are identifiable in many of these cells (9).

In accord with the work just cited concerning other systems, we have postulated that the activation of the cellular contractile mechanism is one of the necessary processes in the movement of neutrophils induced in response to a chemotactic stimulus (10). Our initial approach to obtaining evidence for this hypothesis has been directed to seeing whether glycerinated rabbit peritoneal polymorphonuclear leukocytes (heterophils, neutrophils) will contract when stimulated by exogenous ATP. As the report will show, we have succeeded in demonstrating this contraction as well as relaxation under appropriate circumstances. As in other studies, these phenomena were demonstrated by microscopic observation of single cells. Because this method of observation does not readily lend itself to measurement of the extent of the responses obtained, we have also determined the cell volume. We have demonstrated that associated with the contraction and relaxation observed microscopically there are measurable changes in cell volume. Furthermore, we have obtained evidence that Ca^{2+} is required for the contraction, as observed microscopically, as well as the volume response of glycerinated neutrophils.

Materials and Methods. The rabbit peritoneal neutrophils were obtained as described before (11). The Ca–glycerol buffer used throughout was 0.19*M* glycerol, 0.04*M* KCl, 0.01*M* Tris. Cell morphology and contractile response were observed in a perfusion chamber using a Zeiss phase contrast photomicroscope. The construction of perfusion chamber and perfusion experiments were done according to Simard-Duquesne and Couillard (12) except: (1) glass wool was omitted and cells were allowed to settle and adhere to the glass slide before starting perfusion; (2) ATP and other test solutions were introduced from both open ends of the perfusion chamber in order to minimize the streaming of perfusion liquid. The frequency distribution of the cell volume was determined on a Coulter Model Z particle counter. The instrumental settings were adjusted for each cell preparation so that the control distribution had a mean cell threshold of about 45. Cell counts within a threshold

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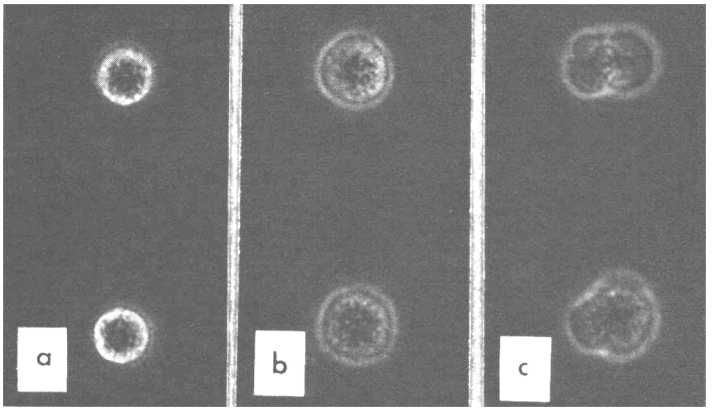

FIG. 1. The swelling of leucocytes in calcium-glycerol buffer (700 ×); (a) leucocytes in peritoneal exudate, (b) 10 min after perfusion with calcium glycerol buffer, (c) 20 min in calcium glycerol buffer.

window of 5 division were determined sequentially starting from a lower threshold setting of 15 to an upper threshold setting of 95. To achieve rapid counting, a 100 μl manometer tube was used and each cell count was completed in five seconds. Cell counts obtained in each threshold window were converted to the percentage of the total cell count and these were plotted against the corresponding number of the threshold window setting to give a frequency distribution curve of cell volume. The number of the threshold window is proportional to the cell volume. All experiments were performed at room temperature.

Results and Discussion. The neutrophils in the peritoneal exudate appeared as rounded and phase dense cells containing cytoplasmic granules (Fig. 1A). After 10 min perfusion with Ca–glycerol buffer the cells became large, rounded and phase lucent and markedly swollen with lysosomal granules which were highly prominent and showed vigorous Brownian movement (Fig. 1B). After approximately 20 min these cells began to spread on the slide and assume various shapes (Fig. 1C).

Ten min after the perfusion of cells with Ca–glycerol buffer (Fig. 2A), a solution of ATP (1m*M*) in Ca–glycerol buffer was intro-

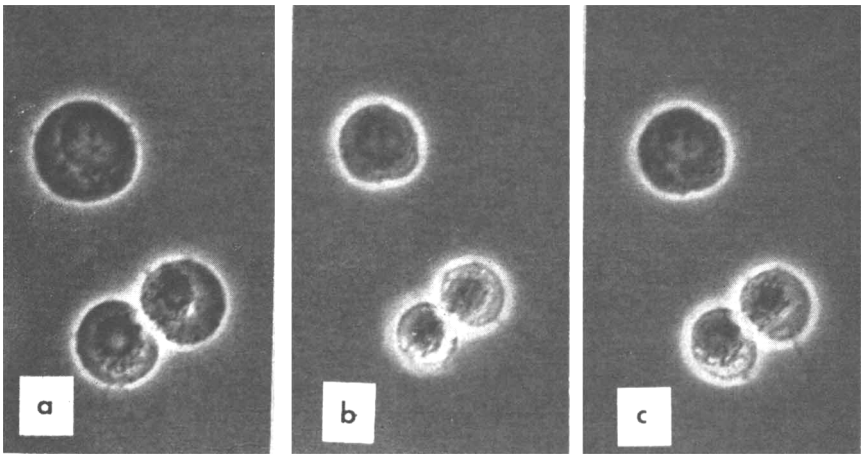

FIG. 2. Contraction of leucocytes by ATP (1 m*M*) in calcium glycerol buffer (840 ×); (a) 10 min after perfusion with calcium glycerol buffer without ATP, (b) 1 min after ATP perfusion, (c) 3 min after ATP perfusion.

duced into the perfusion chamber. Some cells immediately contracted but many cells contracted only after a latent period varying from seconds to over 1 min. The strong radial contraction of cells seen as a rapid diminution of cell area with increasing phase density (Fig. 2B) took only 5–10 sec to complete. Most cells remained in the contracted state for a prolonged period of time; however, there were cells that spontaneously relaxed after one to 2 min following their contraction by ATP (Fig. 2C). Approximately 80% of neutrophils under observation showed contractile responsiveness to ATP as described above. Higher concentration of ATP (>4m*M*) induced a stronger contraction but this was invariably followed by immediate and extensive relaxation of the cells. These observations are very similar to those reported for the contraction of glycerinated amoebae (12). We have not observed a formation of pseudopod-like herniation during the contraction as observed by Norberg (13) in glycerinated monocytes.

Equivalent concentration of AMP and EGTA (ethylene glycol-bis (β-aminoethyl ether) *N-N'* tetraacetic acid) (1m*M*) were both ineffective in causing significant cell contraction suggesting that ATP induced contraction of cells is not caused by nonspecific effects such as increase in osmolarity (3 mOsm/m*M* ATP), ionic strength or chelation of divalent metal ions.

The contractile response induced by ATP was much reduced if cells were first perfused with glycerol buffer containing EGTA (5m*M*) and Mg^{2+} (2m*M*). This suggests that the contraction of the cells in response to ATP requires Ca^{2+}. If Ca^{2+} was not rigorously depleted by using EGTA, however, ATP could induce significant cell contraction in the presence of Mg^{2+} (2 m*M*) alone. The latter observation is essentially the same as previous reports which showed that ATP and Mg^{2+}, in the absence of added Ca^{2+}, induced a contraction of glycerinated cells (1, 12, 13). We further observed that subsequent to the addition of ATP, introducing EGTA (5 m*M*) to the perfusion chamber invariably led to immediate relaxation of the contracted cells suggesting that Ca^{2+} also played a role in the relaxation mechanism.

Similar experiments were carried out with cells in suspension in order to see whether

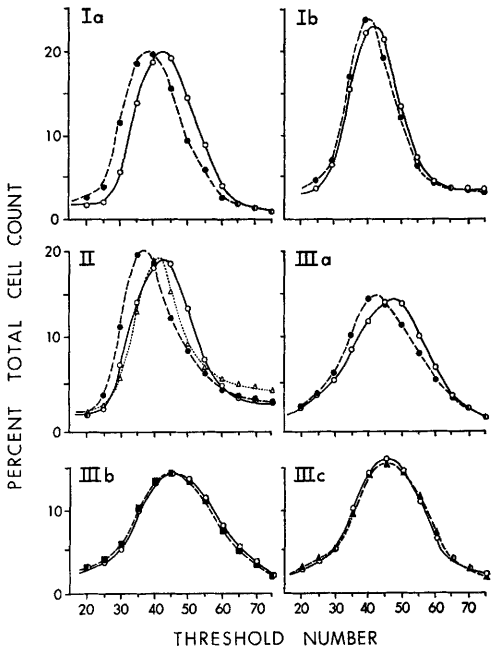

FIG. 3. Effect of ATP, AMP and EGTA on the volume distribution of neutrophils. Cells from three different rabbits were used in Experiments I, II and III. Cells at a concentration of 1 × 10^5/ml were suspended and stirred in Ca-glycerol buffer (Ia, II & $III_{a,b,c}$) or glycerol buffer containing EGTA (5 m*M*), and Mg^{2+} (4m*M*) (I_b) for 20 min before adding ATP (I_a, II & III_a), AMP (III_b) or EGTA (III_c) to a final concentration of 1 m*M*. O—O, control volume distribution; ●---●, 5 min after adding ATP ($I_{a,b}$, II & III_a); ■---■, 5 min after adding AMP (III_b) ; ▲---▲, 5 min after adding EGTA; △····△ (II), EGTA (0.1*M*, pH 8.1) added to a final concentration of 5 m*M* five minutes after the addition of ATP.

there was also a change in the cell volume. Fig. 3 shows representative results using 3 cell preparations from 3 rabbits. The volume distribution curves for neutrophils in Ca–glycerol buffer are apparently skewed and analysis revealed that these curves followed the log-normal rather than the Gaussian distribution. In all of these cells, ATP (1m*M*) caused a shift in the volume distribution (Fig. 3, Ia, II and IIIa) which corresponds to a decrease of mean cell volume of 8.0%, 11.0%, and 5.4%, respectively. If no Ca^{2+} was added and the contaminating Ca^{2+} was chelated by EGTA (5 m*M*) in the presence of Mg^{2+} (4 m*M*), the effect of ATP in causing a decrease of cell volume was markedly inhibited but not

abolished (Fig. 3, 1b.). The residual volume decrease induced by ATP in the presence of EGTA, although small, was repeatedly seen. If EGTA (5 m*M*) was added to Ca–glycerol buffer after ATP, a reversal of the shift of volume distribution, i.e. reexpansion of cell volume was observed (Fig. 3, II). Thus, the ability of EGTA to inhibit and also reverse the ATP effect on cell volume suggests that Ca^{2+} plays a modulatory role in volume response. Control experiments using AMP (1 m*M*) and EGTA (1 m*M*) in place of ATP showed no significant volume response of neutrophils in Ca–glycerol buffer (Fig. 3, IIIb, c).

The morphological observations of the response of neutrophils to exogenous ATP in Ca–glycerol buffer are similar to the reported contraction of glycerinated amoebae (12) and monocytes (13). As with these cells, the contraction of neutrophils was seen as a rapid reduction of cell area, preceded by a latent period, and at high ATP concentrations, followed by extensive relaxation of the cells. However, neutrophils were exposed to Ca–glycerol buffer which is a hypotonic and low ionic strength medium containing only 0,19*M* (1.7%) glycerol for a brief interval (10 min) in contrast to the previously reported glycerinated cells (1, 12, 13) which were extracted with up to 50% glycerol in the cold for hours or days. The ability of exogenous ATP to induce a contraction in the heavily glycerinated cells is presumably due to the accessibility of ATP to the contractile elements of the cells whose surface membrane had been damaged by glycerol extraction. In Ca–glycerol buffer there is also damage of the surface membrane of neutrophils as evidenced by the fact that 30%–50% of lactic dehydrogenase, a cytoplasmic enzyme marker, and β-glucuronidase, a lysosomal enzyme marker, appear in the extracellular fluid within 30 min after the neutrophils are incubated in Ca–glycerol buffer (E. L. Becker, unpublished data). The neutrophils in Ca–glycerol buffer exhibit no spontaneous contraction or any form of cell movement except when ATP is added in the presence of Ca^{2+} or Mg^{2+}. In these respects, the neutrophils in Ca–glycerol buffer are similar to the glycerinated cells which had been extensively used as contractile cell models.

The mechanism whereby ATP and Ca^{2+} decrease the cell volume remains to be investigated. Changes in cell volume are presumably due to changes in the flux of water and/or electrolytes and these could have many causes. What is of interest at this time is that the changes of cell volume induced by ATP and Ca^{2+} parallel the contraction and relaxation of individual cells observed under the microscope. This parallelism is evidenced by the fact that ATP is effective while AMP and EGTA are ineffective in provoking a cell contraction and volume decrease; that EGTA in the presence of Mg^{2+} inhibits the effect of ATP; that EGTA added after ATP reverses the ATP induced cell responses in terms of contraction and volume decrease. These observations suggest that the volume changes of cells in response to ATP and Ca^{2+} result from the same contractile mechanism that causes cells to contract or relax. There is no direct evidence in this study indicating that actomyosin-like interactions are involved in the contractile or volume response of the neutrophils. Nevertheless, in view of the analogies to the findings with glycerinated muscle fiber and the superprecipitation of actomyosin-like proteins isolated from horse neutrophils (5), further investigation of the possible role of actomyosin-like proteins is obviously warranted.

Although the mechanism of the volume response is still being investigated we have already started to study the relation of changes in the volume of nonglycerinated neutrophils to the activation of cell movement. We have shown that chemotactic factors induce an expansion of the volume of living nonglycerinated neutrophils by as much as 12% and this volume expansibility correlates with the chemotactic responsiveness of the cells (14). In work being prepared for publication we have shown that chemotactic factor, in the presence of cytochalasin B (1 μg/ml), which inhibits chemotaxis (15) but enhances lysosomal enzyme release (14) of neutrophils, causes a rapid (<1 min) decrease of the volume of neutrophils by 7%–12%. Both volume expansion and contraction induced by the chemotactic factor are strongly inhibited by glycolytic poisons such as 2-deoxyglucose and iodoacetate indicating that these are energy dependent processes. These observations suggest an intriguing relationship between volume

change and the activation of cell movement. The direct excitation of contraction and volume change by ATP and Ca^{2+} in nonviable, glycerinated neutrophils represents a simplified system to study the mechanism underlying this relationship.

Summary. Rabbit peritoneal polymorphonuclear leucocytes treated with a hypotonic and low ionic strength solution containing glycerol, Ca^{2+} and KCl are contracted by ATP as observed microscopically. Under the same conditions, ATP induces a decrease in cell volume as measured by a Coulter Counter. The measurement of cell volume response gives parallel results to the contractile events observed microscopically in the following respects: (1) The effect of ATP requires Ca^{2+}; (2) AMP and EGTA both fail to elicit a cell response; (3) Chelation of Ca^{2+} causes a reversal of the contractile and volume responses elicited by ATP and Ca^{2+}. From these parallel observations it is suggested that the microscopically observed contraction of glycerinated polymorphonuclear leukocytes induced by ATP and Ca^{2+} is a reflection of a generalised decrease in cell volume.

1. Hoffman-Berling, H., *in* "Comparative Biochemistry" (M. Florkin and H.S., Mason, eds.), Vol. 2, p. 341. Academic Press, New York (1960).

2. Ohnishi, V.T., Kawamura, H., and Tanaka, Y., J. Biochem. **56**, 6 (1964).

3. Bettex-Galland, M., and Lüscher, E.F., Biochim. Biophys. Acta **49**, 536 (1961).

4. Weihing, R.R., and Korn, E.D., Biochemistry **10**, 590 (1971).

5. Senda, N., Shibata, N., Tatsumi, N., Kondo, K., and Hamada, K. Biochim. Biophys. Acta **181**, 191 (1969).

6. Adelstein, R.S., Conti, M.A., Johnson, G.S., Pastan, I., and Pollard, T.D., Proc. Natl. Acad. Sci. **69**, 3693 (1972).

7. Puszkin, S., Berl, S., Puszkin, E., and Clarke, D.D., Science **161**, 170 (1969).

8. Poisner, A.M., Fed. Proc. **29**, 545 (1970).

9. Ishikawa, H., Bischoff, R., and Holtzer, H., J. Cell Biol. **43**, 312 (1969).

10. Becker, E.L., J. Immunol. 106, 689 (1971).

11. Becker, E.L., J. Exp. Med. **135**, 376 (1972).

12. Simard-Duquesne, N., and Couillard, P., Exp. Cell Res. **28**, 85 (1962).

13. Norberg, B., Exp. Cell Res. **59**, 11 (1970).

14. Showell, H.J., Hsu, L.S., and Becker, E.L., Fed. Proc. **32**, 973 (1973).

15. Becker, E.L., Davis, A.T., Estenson, R.D., and Quie, P.G., J. Immunol. **108**, 396 (1972).

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