Effects of an Ionophore, A23187, on the Surface Morphology of Normal Erythrocytes

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A23187, an ionophore which selectively transports divalent cations across biologic membranes, was found in previous studies to cause exchange of calcium for hydrogen ions in erythrocytes, loss of potassium, and a decrease in cell volume due to escape of water without hemolysis. The present investigation has evaluated the influence of A23187 on erythrocyte morphology in the scanning electron microscope. The findings demonstrate that A23187 causes a time- and concentration-dependent conversion of biconcave erythrocytes into echinocytes and spheroechinocytes. The changes induced in erythrocytes by the ionophore required the presence of extracellular calcium. Red cells exposed to ionophore at low temperature were moderately resistant to its effects, and low pH (5.5 or less) virtually blocked the action of the drug. The findings support an important role for calcium in maintaining the discoid configuration of normal erythrocytes (Am J Pathol 77:507–518, 1974).

A23187 IS AN IONOPHORE which selectively transports divalent cations across biologic membranes.[1–3] The agent has been used to evaluate the influence of an artificial increase in calcium ion concentration in secretory cells and in isolated subcellular organelles.[4–6] Effects of the ionophore on red blood cells have also been investigated.[7] A23187 caused the exchange of extracellular calcium for erythrocyte hydrogen ions, a loss of cell water, and a concomitant extrusion of potassium without inducing hemolysis. The action of the agent on erythrocyte morphology was not reported.

We have recently explored the effects of A23187 on blood platelet structure and function [8,9] and as an effector of mitogenesis in lymphocytes.[10] During the course of these studies specific effects of the agent on erythrocyte morphology were observed. The present investigation was initiated to determine the nature of the structural changes induced in erythrocytes by the ionophore.

Materials and Methods

The technics used in this laboratory to obtain blood from normal donors; mix the samples immediately with 3.8% trisodium citrate or citrate-citric acid, pH 6.5,

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in a ratio of 9 parts blood to 1 part anticoagulant; separate platelets and leukocytes from red blood cells by differential centrifugation at room temperature; expose blood cells to various drugs and experimental conditions *in vitro*; and fix control and experimental samples for study in the light, phase contrast, transmission and scanning electron microscopes have been described in detail in several recent reports.[11–13] Procedures employed in the present investigation were identical. Erythrocytes separated from whole blood were washed three times and resuspended in Hanks' buffered salt solution (HBSS), pH 7.3, containing 0.1% bovine serum albumin. For some experiments the red cells were washed and resuspended in HBSS devoid of calcium and magnesium ions. A23187 (kindly supplied by Robert D. Hamill, PhD, of the Eli Lilly Research Laboratories, Indianapolis, Ind) was dissolved in dimethyl sulfoxide (DMSO) or 95% ethanol at a concentration of 1 mg/ml and kept frozen between experiments. The stock solution of A23187 was diluted to ten times the desired final concentration in 0.3 M Tris-buffered saline, pH 7.3. A 0.1- or 0.05-ml volume of the agent was added to 0.9- or 0.45-ml volumes of washed erythrocytes, respectively. Samples of erythrocytes were incubated with A23187 at final concentrations of 2, 10, 20, 100 and 200-μM for intervals of 1, 3, 5, 8, 10, 15, 30 and 60 minutes at 37 C. Additional samples of red cells suspended in HBSS without calcium or magnesium were combined with the ionophore at concentrations of 10 or 20 μM and incubated for 15 minutes at 37 C. $CaCl_2$ (1.25 mM), $MgCl_2$ (.84 mM), EDTA (2 mM) and combinations of $CaCl_2$ plus EDTA or $MgCl_2$ and EDTA were added to separate samples of washed erythrocytes suspended in divalent-cation-free HBSS with either of the two concentrations of ionophore for 15 and 30 minutes. Additional erythrocytes were washed and resuspended with the ionophore in samples of HBSS, each at a different pH. The pH was adjusted to a range of 5.5 to 9.0 by dropwise addition of 1 N HCl or 1 N NaOH and was readjusted as necessary by periodic measurements during incubation of erythrocytes with A23187 for 15 to 30 minutes. Some samples of washed erythrocytes were incubated with 10 or 20 μM calcimycin at 4 C and 24 C, as well as at 37 C.

Reversal of ionophore effects was attempted by combining washed erythrocytes with 10 or 20 μM calcimycin in HBSS containing Ca^{++} and Mg^{++} for 15 minutes, washing 3 times in HBSS without divalent cations and incubating an additional 15 to 30 minutes in divalent-cation-free HBSS. All of the control and experimental samples of erythrocytes were fixed for study in the Cambridge Stereoscan S4-D10 scanning electron microscope (SEM) according to the schedule described previously.[13] After fixation and dehydration through a series of alcohols, the samples were dried by the critical point method of Anderson [14] and coated with a 50- to 100-Å layer of gold in an Edwards vacuum evaporator.

Results

Control Erythrocytes

The biconcave discoid form of erythrocytes was well preserved in control samples examined in the scanning electron microscope (SEM) (Figure 1A). Erythrocytes in whole blood, in plasma, or washed and resuspended in HBSS with 0.1% albumin with or without added calcium and magnesium ions retained their unaltered shape. Exposure of washed erythrocytes in HBSS with 0.1% albumin to DMSO or ethanol alone diluted to the maximum concentration (1% or less) added with A23187

to experimental samples did not influence the biconcave appearance of control cells after 15 to 30 minutes of incubation at 37 C.

General Effects of A23187

The influence of A23187 on erythrocyte forms was first noted in thin sections of cells from whole blood and plasma and was easily produced in samples of washed erythrocytes. Red cells incubated 15 to 60 minutes with A23187 at final concentrations of 10 μM or greater were converted from biconcave discocytes into echinocytes or spheroechinocytes. The number of cells manifesting significant alterations in form and the degree of shape change varied with the concentration of the agent and duration of exposure to it (Figure 1B–D). All of the erythrocytes combined with 100 to 200 μM ionophore for 15 minutes were spheroechinocytes (Figure 2G and H). A concentration of 20 μM produced the same degree and extent of change in erythrocyte form after 30 to 60 minutes of incubation. Samples combined with A23187 at 2 μM did not undergo significant alterations in shape after incubation for intervals up to 1 hour.

Time Dependence of A23187 Influence

The rate of shape transformation was observed at intervals in samples of washed erythrocytes exposed to A23187 at concentrations of 10 or 20 μM. Shape change was evident in erythrocytes combined with 20 μM A23187 by 3 minutes but was not apparent until 8 minutes after exposure to 10 μM. The sequential alterations leading ultimately to the spheroechinocyte could be identified in the samples exposed for increasing intervals to 10 μM A23187.

The first change evident in the surface topography of red cells following exposure to the ionophore appeared to be the development of small blebs randomly dispersed on the biconcave discs (Figure 1E and F). On some cells the dimples were extruded into slender stalks. Shape change was initially manifested by an irregular distortion, as if the discocyte had been twisted (Figure 1F–H). A more advanced stage was signaled by development of a variable number of short, cone-shaped extrusions on the discoid cells (Figures 1H, 2A and 2B). This alteration was followed by loss of the central concavity and conversion to an oval or spherical form covered with cone-shaped spikes (Figure 2C–F). Longer exposure to A23187 resulted in the appearance of more and more cells that appeared smaller in size, spherical in form, and covered with large numbers of long tubular extrusions (Figures 2G, 2H and 3A-H). The spheroechinocyte seemed to be the final stage

of transformation induced by A23817 in most cells, but a few were changed into spheres without surface extensions. Relative numbers and the length of the tubular extensions on ionophore-induced spheroechinocytes varied after longer exposure to the ionophore or incubation with higher concentrations of the agent, often appearing relatively blunt on the cells exposed to a more severe stress (Figure 4A–D).

Reversibility of A23187-Induced Transformation

Efforts to reverse the effects of A23187 by washing cells incubated with the agent long enough (15 minutes) to produce spheroechinocytes and resuspending them in plasma or HBSS without calcium and magnesium ions were unsuccessful. Once the state of the spheroechinocyte had been reached, it did not appear to change under the conditions of these experiments.

Effects of Divalent Cations on A23187-Induced Transformation

Erythrocytes washed and resuspended in HBSS containing 0.1% albumin but without added calcium or magnesium ions developed typical changes in about half the cells after 15 minutes incubation with A23187 at a concentration of 10 μM. When EDTA was added before A23187, all of the cells remained discoid under the same conditions. Erythrocytes suspended in HBSS containing calcium, but not magnesium, ions were uniformly changed into spheroechinocytes 15 minutes after exposure to 10 μM A23187. EDTA added with calcium before the ionophore completely blocked the shape change. Red cells in HBSS containing magnesium ions, but devoid of added calcium, were all discoid 15 minutes after incubation with 10 μM ionophore. The preservation of discoid shape in the presence of A23187 and Mg^{++} ions was unaffected by EDTA.

Influence of Temperature and pH

Erythrocytes incubated with A23187 (10 μM for 15 to 30 minutes at 4 C or 24 C retained their discoid form or appeared cup-shaped, while cells exposed to the same amount of ionophore for the same period of time at 37 C were uniformly transformed into spheroechinocytes. The 20 μM concentration of A23187, however, caused discocyte–echinocyte transformation at both 24 C and 4 C.

Samples of erythrocytes incubated with A23187 at a pH of 5.5 retained their biconcave form but developed small surface blebs and slender stalks (Figure 4E–H). Each increase of incubation pH above 5.5 resulted in a greater degree of surface alteration induced by the

ionophore. All of the cells exposed to A23187 at pH 9 were spheroechinocytes covered with long or blunt surface projections.

Discussion

The present investigation has demonstrated that A23187, an ionophore which selectively transports divalent cations across biologic membranes until equilibrium is reached,[1-7] causes the transformation of biconcave discocytes into spheroechinocytes. Erythrocyte shape change induced by the ionophore was concentration and time dependent, occurring more rapidly and completely in the presence of increasing amounts of the agent. Calcium ions in the suspending medium appeared to be essential for induction of shape change. Some transformation of erythrocytes to echinocytes was noted in washed discocytes suspended in HBSS without Ca^{++} or Mg^{++}, but the 0.1% albumin may have supplied enough divalent cation to support the conversion. Shape change was blocked in the presence of EDTA even when divalent cations were present in the medium. Calcium in the absence of magnesium supported A23187-induced transformation, while magnesium in the absence of calcium did not. In fact, changes were more apparent in cells exposed to A23187 in the absence of divalent cations than in the presence of added magnesium. A similar requirement for calcium ions in the suspending medium has been observed in other cell systems responding to stimulation by the ionophore.[5,6]

Since A23187 is a monocarboxylic acid,[1-3] its effects on red cells might have been related to pH change, even though it decreased the pH of suspended cells by 0.2 pH units or less. Examination of the influence of pH on A23187-induced erythrocyte alterations indicated that this was unlikely. Low pH inhibited conversion to the spheroecchinocyte form, while high pH fostered the transition. Previous studies have shown that high pH promotes the development of echinocytes and low pH favors the transformation of discocytes to spherocytes.[15-17] The time intervals of incubation at low pH in this study were too short to promote spherocyte formation, but significantly inhibited spheroechinocyte transformation by the ionophore. It is uncertain whether the effect of low pH was due to an inherent resistance of acidified red cells to become echinocytic or to the influence of decreased pH on the ionization of calcium in the medium.[18]

Erythrocytes incubated with ionophore at 4 C or 24 C needed twice as much A23187 to induce shape change as cells maintained at 37 C, and the alteration in morphology was confined largely to the echinocyte stage. Despite decreased sensitivity of cells at room temperature or 4 C,

it is evident that the ionophore can induce physical changes under these conditions. The active metabolic state of erythrocytes at 37 C may render the cells more susceptible to stress imposed by A23187.

The changes in erythrocyte morphology during conversion from discocytes to echinocytes and spheroechinocytes after exposure to A23187 confirms and extends previous observations on this type of phasic red cell transition described by Bessis.[15–17,19,20] Initially, the discocytes developed only slight changes consisting of small dimples and slender stalks. Minute changes of this nature could not be identified in the light microscope but were regularly present on A23187-treated erythrocytes viewed in the SEM. The next stage in transition involved the extension of cone-shaped spikes and knobs over the rim or convex surface of the biconcave cell. The shape of erythrocytes manifesting this alteration was often distorted or twisted, but the central concavity was retained. As the number of cone-shaped spikes on the surface increased, the cells lost their discoid shape and became more spherical, with spikes protruding in all directions. Conversion of this form to the spheroechinocyte covered with long, slender pseudopods must have occurred rapidly, because further intermediate stages in development could not be identified. Although the length of the pseudopods varied on these cells, appearing to be shorter on erythrocytes exposed for longer periods to the agent, the physical changes of the ionophore-induced spheroechinocyte were not reversible.

The transition phases described above are somewhat arbitrary because nearly all degrees of transformation were apparent in the first samples in which any significant change was evident. However, the multiple observations on samples exposed to various concentrations of ionophore for increasing time intervals under several conditions of pH and temperature indicate that the suggested sequence of physical alteration caused by A23187 is reasonable. One of the most interesting changes was the transition from an echinocyte covered with cone-shaped spiky projections to the spheroechinocyte with long tubular pseudopods. The spheroechinocytes appear grossly reduced in size, probably due to loss of water as previous studies have suggested.[7] Loss of cell volume may be related to sphere formation and to the conversion of cone-shaped spikes to long tubular elements. If this is the case, then it may be possible to relate a distinct morphologic transition to a specific event, the loss of cell water. Experiments in progress have confirmed that A23187 causes red cells to lose approximately 20% of their volume,[21] and efforts are currently in progress to relate the volume loss to a specific stage in ionophore-induced spheroechinocyte formation.

Earlier studies by Weed and Chailley have demonstrated that exposure of erythrocytes to calcium concentrations of 10^{-2} M or higher for periods of several hours at 37 C *in vitro* will also cause an irreversible echinocytic shape change. Observations obtained in the present investigation have confirmed nearly all the findings of these authors. Thus movement of calcium ions into or through the red cell membrane, either as a result of exposure to very high concentrations of calcium for long time intervals or incubation of the cells with an ionophore at normal or low calcium concentrations for brief periods of time, will result in irreversible spheroechinocyte formation. The results further emphasize the important role of calcium in the integrity of the biconcave form of red blood cells and in the response of erythrocytes to agents which deform the cell.[17,22]

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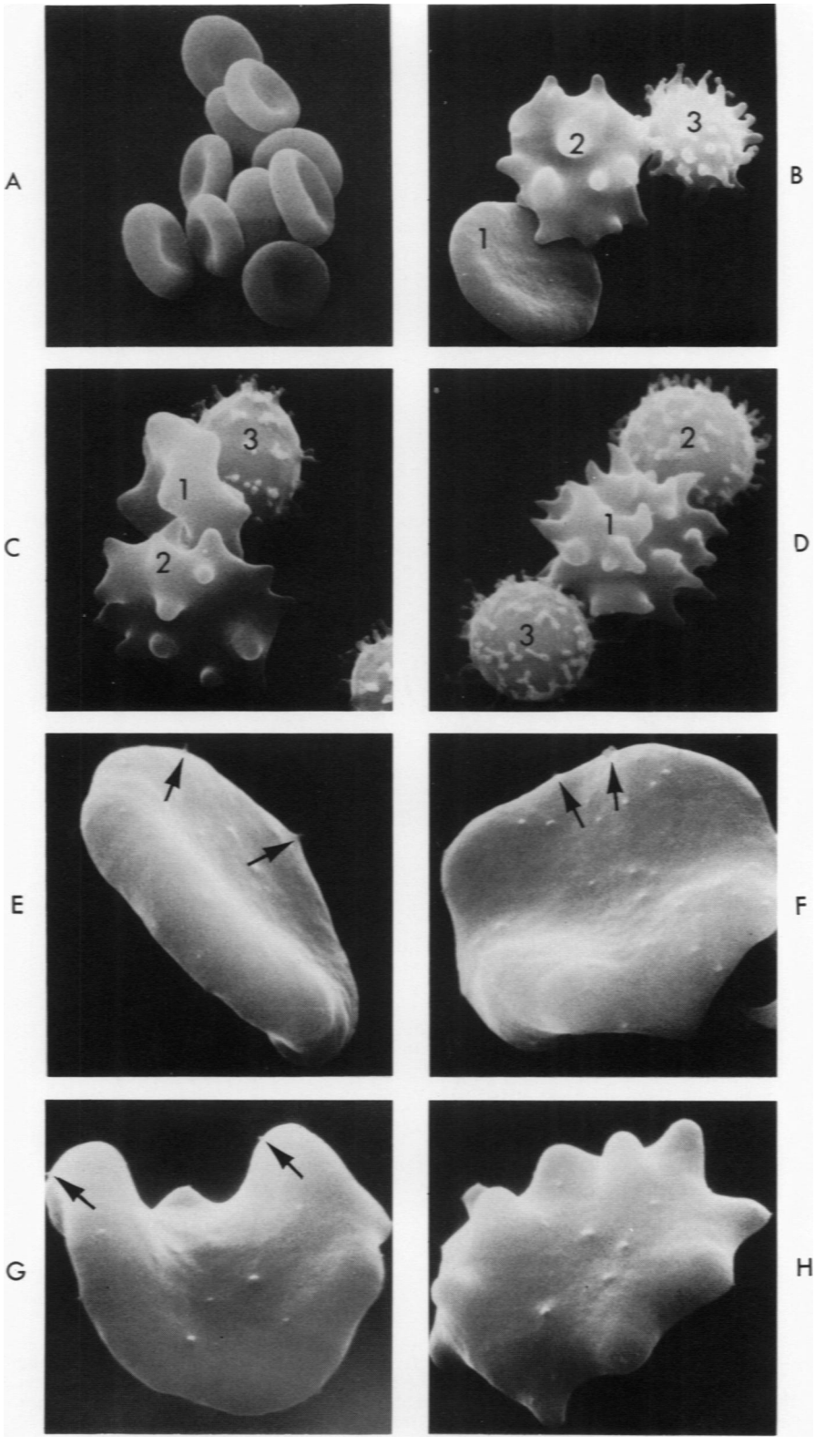
Legends for Figures

Fig 1—Erythrocytes in **A** are from a sample of normal red cells washed and resuspended in HBSS with 0.1% albumin. The biconcave discoid shape of the cells is well preserved. The erythrocytes in **B, C** and **D** were exposed to 10, 20 and 100 μM A23187, respectively, for 15 minutes. In **B**, three cells manifest various degrees of change. One cell (*1*) has developed surface blebs, a second (*2*) appears midway into echinocyte formation, and a third (*3*) has become an early spheroechinocyte. Note that the third cell appears grossly smaller than *1* and *2*. The cells in **C** demonstrate early (*1*) and middle stage (*2*) echinocyte transformation and late spheroechinocyte development (*3*). A late echinocytic form (*1*) and two spheroechinocytes (*2* and *3*) are evident in **D**. Early stages in development of A23187-induced echinocytes are shown in **E** through **H**. Dimples or blebs are evident on the surfaces of each cell, and slender stalks (*arrows*) project from three. The erythrocytes in **F** and **G** are twisted but remain biconcave. The cell in **H** has developed multiple knobs and cone-shaped spikes at its periphery (Original magnifications, **A**, ×2600; **B**, ×6700; **C**, ×6700; **D**, ×6750; **E**, ×13,000; **F**, ×13,000; **G**, ×13,000; **H**, ×13,000).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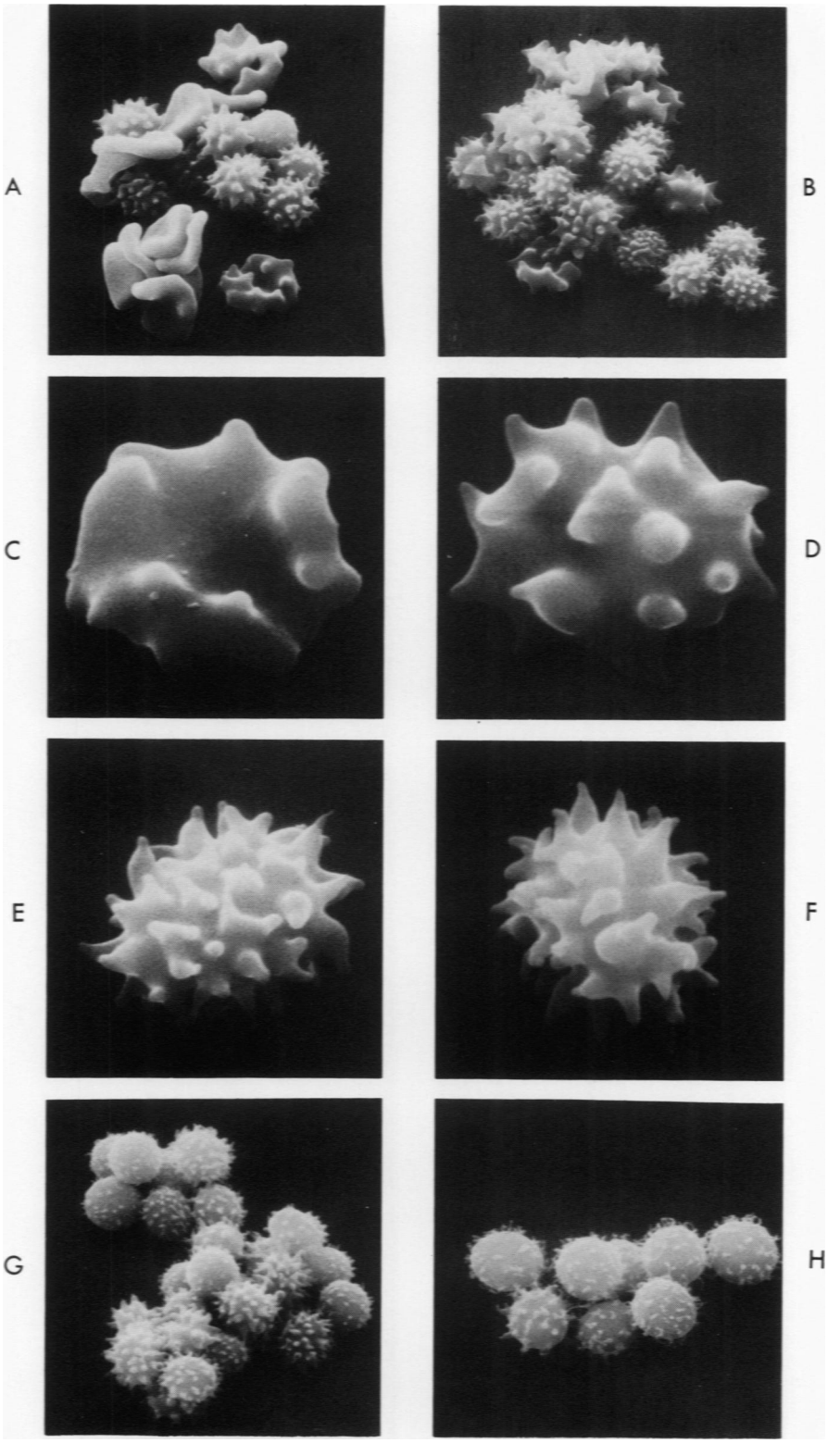

Fig 2—Stages in echinocyte formation and conversion of echinocytes to spheroechinocytes. The cells in **A** were exposed to 10 μM A23187 for 8 minutes, and the erythrocytes in **B** to the same concentration for 10 minutes. Multiple stages in echinocyte transformation can be identified in each photograph. **C–F** trace stages in conversion from early (**C**), to middle stage (**D**) and to late echinocyte forms (**E** and **F**). Erythrocytes in **G** and **H** were exposed to 100 μM A23187 for 15 and 30 minutes, respectively. Most of the cells in **G** and all of the erythrocytes in **H** are spheroechinocytes (Original magnifications, **A**, × 2800; **B**, × 2800; **C**, × 7000; **D**, × 7000; **E**, × 7000; **F**, × 6750; **G**, × 2700; **H**, × 3000).

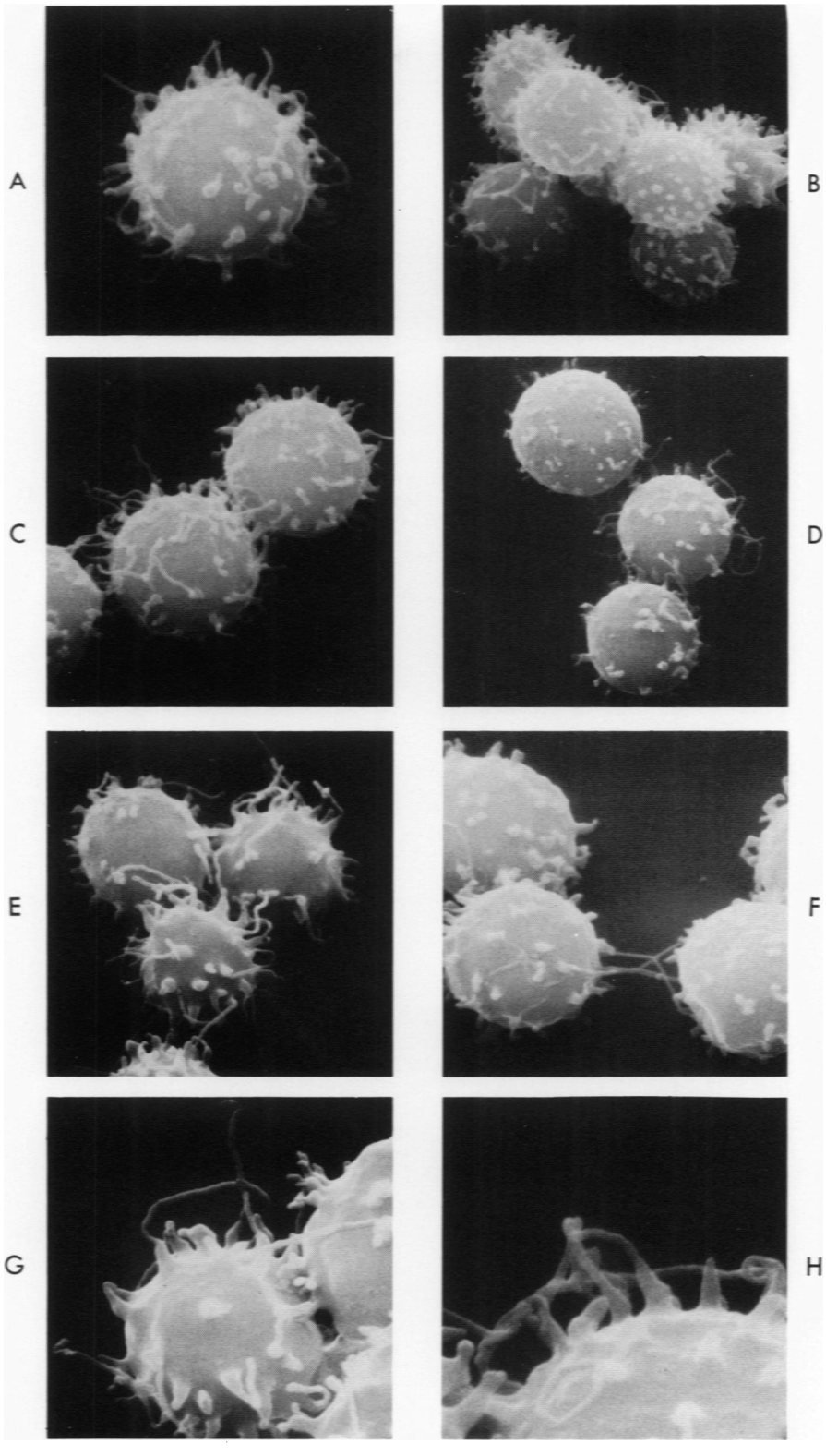

Fig 3—Spheroechinocytes. The cell in **A** was exposed to 10 μ ionophore for 30 minutes, while the cells in **B** were combined with 200 μM A23187 for 10 minutes. Other examples in this illustration are from samples exposed to a sufficient concentration of ionophore long enough that most cells had become spheroechinocytes. Long thin pseudopods extend from the surfaces of many erythrocytes, while the projections on some appear blunt. Pseudopods between two cells in **F** appear to have linked them together (Original magnifications, **A**, × 7500; **B**, × 6500; **C**, × 7500; **D**, × 7000; **E**, × 6500; **F**, × 7500; **G**, × 13,000; **H**, × 27,000).

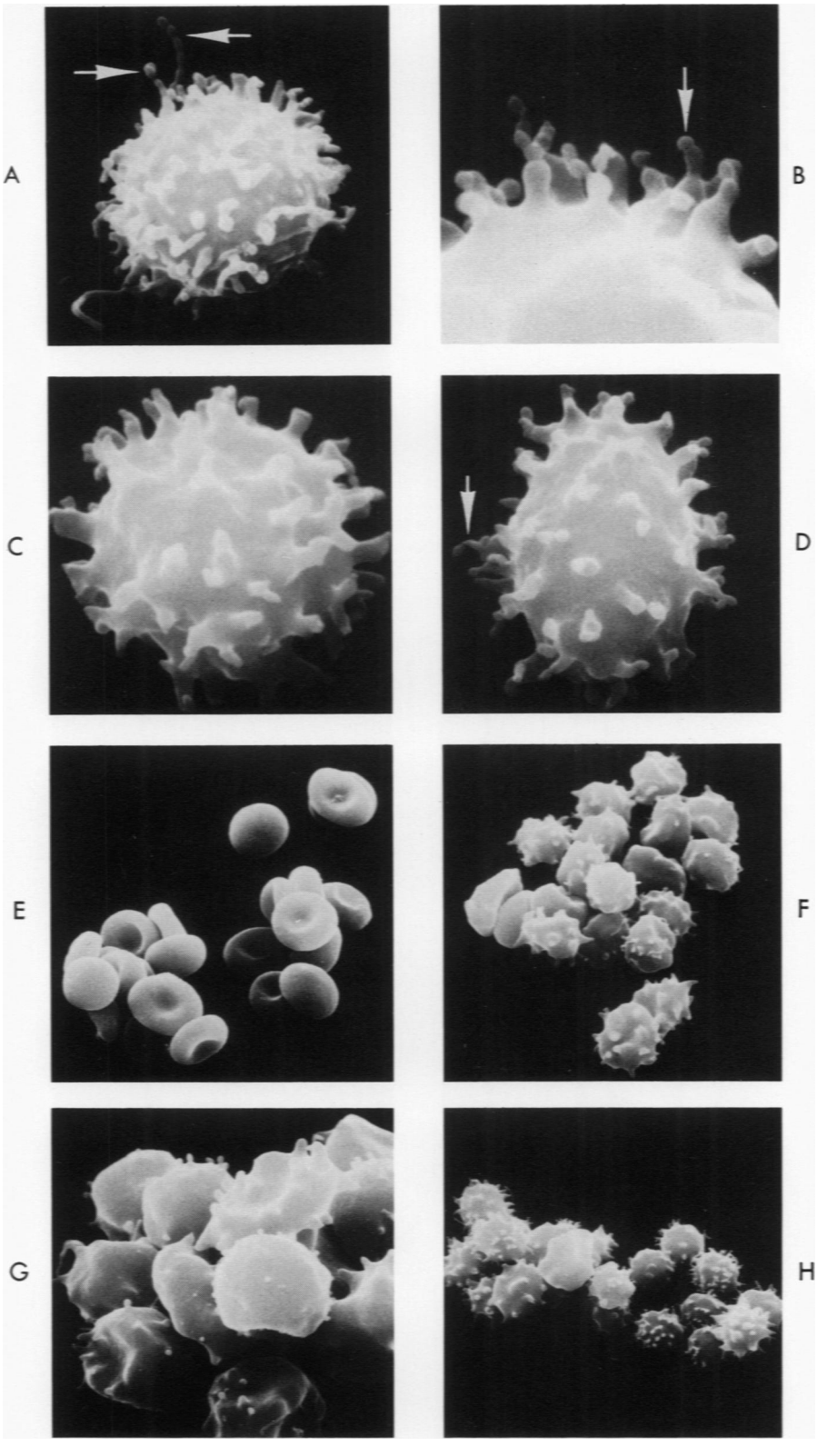

Fig 4—Late stage spheroechinocytes and effects of pH. Spheroechinocytes with blunt surface projections may develop when long pseudopods are pinched off. Constrictions on the long pseudopods (*arrows*) in **A** appear to have formed and may be in the process of pinching off. A similar process can be identified in **B** and **D** and may lead to the late stage spheroechinocytes shown in **C** and **D**. The cells in **E** and **F** were exposed to 10 μM A23187 for 30 minutes at different levels of pH. At a pH of 5.5, some erythrocytes in **E** appear cup-shaped and have dimples and short stalks but otherwise are unaffected. The cells in **F** and **G** were incubated with the agent at a pH of 6 and appear to be somewhat resistent to deformation by the agent. At pH 6.5 the cells in **H** are indistinguishable from erythrocytes incubated at pH 7 to 7.5. Erythrocytes incubated with A23187 at pH of 8 or above (not shown) were all in the echinocytic or spheroechinocyte form (Original magnifications, **A**, × 12,000; **B**, × 28,000; **C**, × 16,000; **D**, × 13,000; **E**, × 2400; **F**, × 2400; **G**, × 6000; **H**, × 2400).