A Circulating Factor(s) Mediates Cell Depolarization in Hemorrhagic Shock

JOHN A. EVANS, PH.D.,* DANIEL N. DARLINGTON, PH.D.,† and DONALD S. GANN, M.D.†

Cell depolarization in hemorrhagic shock has been attributed to hypoperfusion, but the mechanism remains unclear. Suspensions of single cell lines loaded with the potential-sensitive fluorescent dye bis-(1,3-dibutylbarbiturioc acid) trimethine oxonal (DIBAC) and exposed for 30 minutes to rat plasma drawn either before or after hemorrhagic shock (bled 20 mL/kg: mean arterial blood pressure less than 40 mmHg) were studied. Plasma drawn after, but not before, hemorrhage led to partial depolarization regardless of cell type (rat H9C2 skeletal muscle, A-10 smooth muscle, C-9 liver, adrenal, kidney, red blood cell [RBC], white blood cell [WBC]) or species (cat, dog, pig RBC; cat WBC; mouse C2C12 skeletal muscle; and human intestinal smooth muscle [HISM]). Dialysis did not remove the factor(s), suggesting a molecular weight of more than 10,000 daltons. The factor appeared within 5 minutes of shock. The depolarization amplitude increased as a function of plasma concentration and demonstrated saturation kinetics indicating specific receptor binding. Cells were equivalently oxygenated, excluding hypoperfusion as a necessary condition for depolarization. Tumor necrosis factor or platelet activating factor alone or in combination were not effective in this system. Stable measurements can be obtained with this noninvasive system that avoids cell injury consequent to cell impalement with electrodes. This system provides a sensitive *in vitro* bioassay that should permit identification of the plasma factors mediating cell depolarization, as well as definition of the responsible intracellular mechanisms.

From the Departments of Surgery and Biological Chemistry and Physiology,† School of Medicine, University of Maryland at Baltimore, Baltimore, Maryland*

THE PATHOPHYSIOLOGY OF hypovolemic and septic shock is characterized by cell membrane depolarization, that is, a decrease in the absolute value of the transmembrane potential. It has been suggested that the membrane depolarization results from hypoxia, which results, in turn, from hypoperfusion of tissue.[1] Several authors noted the presence of toxic factors that appear after hemorrhage,[2-6] but none have been identified. Studies that applied tourniquet isolation[7] or cross-perfusion[8] suggested the presence of circulating factors mediating depolarization of muscle cells *in vivo*. The use of microelectrodes for recording membrane potential in these and related studies[7,8] has been criticized because of artifacts that may result from puncture of cells.

In the present report we developed a bioassay for a presumptive circulating factor(s) based on the sensitivity of the fluorescence of the membrane dye bis-(1,3-dibutylbarbiturio acid) trimethine oxonal (DIBAC, Molecular Probes, Eugene, OR) to changes in the resting membrane potential and the ability to manipulate such changes by exposure of cells to plasma obtained from rats in shock. This measurement of changes in fluorescence of a membrane-bound dye avoids the problems associated with direct puncture of cells. We used this method to demonstrate the presence of a high–molecular-weight substance(s) that appears in plasma rapidly after hemorrhage and that depolarizes cells derived from many tissues and species, including humans.

Methods

Tissues and Cells

Red blood cells (RBC) were prepared from whole blood by low-speed centrifugation. The RBCs were washed free of residual plasma by centrifugation and 50 uL packed RBCs were resuspended in 5 mL RBC washing solution consisting of 150 mmol/L (millimolar) NaCl; 1 mmol/L MgCl2; 10 mmol/L TRIS-HCI, pH 7.4; 0.1 mmol/L ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA); and 14 mmol/L glucose.[9] White blood cells (WBC) were prepared by differential centrifugation of the

Presented at the 102nd Annual Scientific Session of the Southern Surgical Association, Boca Raton, Florida, December 3–6, 1990.

Supported by NIH grant GM27946.

Address reprint requests to Dr. Donald S. Gann, Department of Surgery, University of Maryland at Baltimore, 22 South Greene St., Baltimore, MD 21201.

Accepted for publication January 16, 1991.

'buffy coat' from RBC preparations. For some experiments WBCs were purified further by centrifugation through Lymphocyte Separation Medium (Organon Teknika, Durham, NC). Defined cell lines derived from tissue culture were obtained from American Type Culture Collection (Rockville, MD) and were maintained in culture. Rat kidney and adrenal tissue were harvested 2 hours after hemorrhage (described below) or from nonhemorrhaged controls. The tissue was dissociated with collagenase and the epithelial cells were harvested and cultured according to the method of Engeland et al.[10] Dog adipose tissue was dissociated with collagenase and the adipocytes were cultured under the same conditions used for the kidney and adrenal tissues.

Preparation of Rats and Plasma

Male Sprague-Dawley rats, weighing 300 to 400 g, were anesthetized with pentobarbital-sodium (50 mg/kg, administered intraperitoneally) and femoral venous and arterial cannulae (PE-50, Dural Plastics, Dural, NSW, Australia) were placed using sterile procedures previously described.[11,12] Cannulae were tunneled under the skin of the back and brought out through a flexible stainless steel spring attached to the back of the neck. The free end of the spring was attached to the top of the cage and was allowed to rotate freely. Incisions were filled with xylocaine jelly and Bacitracin (Medalist, San Francisco, CA) to desensitize the surgical area and prevent infection, respectively. All rats recovered and were caged singly in a controlled temperature environment. The light cycle was 12 hours of light and 12 hours of dark. All rats had access to food and water *ad libitum*. Cannulae were flushed daily 1 to 3 hours after the beginning of the photoperiod. Hemorrhage was induced (20 mL/kg for 4 minutes) from the arterial cannula in the morning, 1 or 2 hours after lights were turned on. Blood samples (0.5 to 1.0 mL) were drawn over heparin at times 0, 2, 3, 10, 20, 30, 60, 120 minutes after hemorrhage. In some experiments, blood samples were also taken at 15, 30, 60, 90, 150, and 210 seconds. Blood samples taken between 0 and 4 minutes were derived from the hemorrhage volume. Blood samples were centrifuged and the plasma was removed and put on ice. Rats were killed after 2 hours by overdose of pentobarbital (100 mg/kg). Plasma was assayed on the day of collection. These experimental protocols were approved by the Committee on Animal Care and Use at the University of Maryland, Baltimore, MD.

Fluorescence Assay of Membrane Potential

Bis- (1,3-dibutylbarbituric acid) trimethine oxonol is a member of the oxonol class of membrane dyes in which a negative charge is delocalized over a generally planer molecule. Oxonol dyes behave much like tetraphenyl boron and triphenylmethyl phosphonium ions and partition into hydrophobic regions of membranes with consequent fluorescence enhancement.[13] This tendency to partition from the extracellular medium into the membrane is countered by the magnitude of the negative interior of the cells, thus providing a means to measure the relative cell polarity.

Except as noted, RBCs and WBCs were incubated and observed in Na/K buffer consisting of 125 mmol/L NaCl; 5 KCl; 23 mmol/L NaHCO3; 0.8 mmol/L MgCl2, 1.8 mmol/L CaCl2; and 20 mmol/L HEPES, pH 7.4. Red blood cells (50 μL) were mixed with 50 μL plasma or buffer and 100 μL buffer containing 300 nmol/l (nanomolar) DIBAC and incubated for 30 minutes at room temperature. Red blood cells were washed free of plasma by addition of 1.5 mL buffer containing 150 nmol/L DIBAC and pelleted in an Eppendorf centrifuge. The cells were resuspended in 3.0 mL buffer with 150 nmol/L DIBAC and the fluorescence was measured in counts per second(cps) using a Spex Industries (Edison, NJ) ARCM Cation Measurement spectrofluorometer. Specimens were excited by a 150-watt xenon lamp filtered to a 10-nm band width about 490 nm by a Corion P10-490-F-F519 filter; emission was observed through a 10-nm band width about 510 nm *via* a P10-510-F-P558 filter (Corion Corp., Holliston, MA). Other cell types were counted with trypan blue and suspended in RBC washing solution such that 50 μL contained approximately 500,000 cells. The remainder of the procedure was similar to that for RBCs, except for a lower *g*-force centrifugation (half speed IEC model UV centrifuge) was used to wash cells free of plasma. For some experiments cells were washed by layering over 2 mL of 10% Hypaque (Winthrop Labs, New York, NY) in Na/K buffer containing 150 nmol/L DIBAC and centrifuging at low speed. We observed approximately a 1% increase in the fluorescence amplitude per millivolt depolarization, which is consistent with published observations.[14]

Reagents

Platelet activating factor (PAF) was obtained from Calbiochem (La Jolla, CA). Human tumor necrosis factor(alpha) (TNF) and endothelins 1, 2, and 3 were obtained from Bachem (Torrance, CA). High-performance liquid chromatography (HPLC) grade water from a RGW-5 system (Photronix Corp., Medway, MA) was used in all solutions. All other reagent, and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

Shires and associates[1] previously showed that hemorrhage leads to a depolarization of muscle cells *in vivo* as measured by microelectrodes. To determine if hemor-

rhage will depolarize cultured cells in an *in vitro* system using the fluorescent dye, DIBAC, we challenged renal epithelial cells (derived from renal tissue that was removed 2 hours after hemorrhage) with plasma taken before and 2 hours after hemorrhage (control and shock plasma). In a single experiment, we found that the fluorescence intensities were 21,500 and 57,100 cps for control and shock plasma, respectively. The ratio of intensities was 2.65. We also found that the renal epithelial cells derived from nonhemorrhaged rats also responded to control and shock plasma (12,500 and 57,100 cps, respectively, for a single experiment; the ratio of intensities was 3.40), thereby demonstrating that the tissue need not be exposed to shock before harvesting to show depolarization when incubated with shock plasma.

To determine if the difference in fluorescence intensity between cells exposed to control and shock plasma results from a differential retention of plasma protein, renal epithelial cells were incubated with plasma, washed, and resuspended in fresh buffer. Under these conditions, we found that the fluorescence intensity from cells exposed to control plasma or shock plasma was 93,900 and 145,300 cps, respectively (in a single experiment), suggesting that the difference in intensity does not result from retention of plasma. However, because the intensity ratio (1.55) was found to be smaller than those in the two previous experiments, these results also suggest that plasma proteins artifactually enhance fluorescence if not removed by washing. Because of these findings, all subsequent experiments were performed after washing cells free of any residual plasma. Cells not derived from cultured cell lines were obtained from control (nonhemorrhaged) rats.

Species and Cell Type Specificity and Ionic Composition of Buffer

Plasma taken 1 hour after hemorrhage depolarized RBCs from rat, cat, dog, and pig (Table 1) smooth and skeletal muscle, liver, kidney, and adrenal from rat; dog adipocytes; mouse skeletal; and human intestinal smooth muscle (Table 2). We also found that dog and pig RBCs depolarized to shock plasma only when washed and incubated in a high Na^+ buffer (Table 1). Substitution of K^+ for Na^+ prevented depolarization (Table 1), suggesting that Na^+ ions are essential. Depolarization occurred when the hydrogen ion concentration of the buffer was adjusted to pH 7.4, suggesting that acidosis is not necessary for depolarization by shock plasma.

TABLE 1. *Red Blood Cell Response to Shock Plasma*

Species	Ratio
Rat	1.20
Cat	1.24
Dog	1.68
Dog	1.03(*)
Pig	1.80
Pig	1.08(*)

Incubation and observation was in sodium buffer (mmol/L [millimolar]): 125 NaCl; 23 NaHCO3; 0.8 MgCl2; 1.8 CaCl2; 1 NaH2PO4; 20 HEPES pH 7.4), except as noted (*), which was in potassium buffer, in which KCl was substituted for NaCl. Ratio is fluorescence intensity after exposure to shock plasma divided by that after control plasma.

TABLE 2. *Response to Shock Plasma*

Cell Type	Ratio
Rat adrenal	1.11
R. a7r5 smooth	1.40
R. C-9 liver	1.15
R. H9C2 skeletal	1.30
R. A-10 aortic	1.40
R. WBC	1.52
Mouse C2C12 skeletal	1.17
HISM	1.12
Cat WBC	1.19(*)
Dog adipocyte	1.46

Conditions were as given in Methods using Na/K buffer, except (*) used sodium buffer as in Table 1.
WBC, white blood cell; HISM, human intestinal smooth muscle.

Time Course of Appearance of the Factor(s)

Plasma taken at various times after 20 mL/kg (for 4 minutes) hemorrhage causes depolarization of rat RBCs and rat a7r5 smooth muscle cells that reaches a maximum at approximately 20 minutes followed by a gradual decline (Fig. 1). The ordinate is expressed as the percentage change in fluorescence intensity because this parameter is linear with change in resting membrane potential.[14] The response determined with a7r5 smooth muscle cells is similar to the response of RBCs. However the effect persists longer than it does in RBCs.

Dose Response

The fluorescence amplitude caused by depolarization of rat RBCs increases with plasma concentration and then saturates, indicating an equilibrium binding process (Fig. 2). Equilibrium kinetics suggest a receptor-mediated process.

Cytokines

Because of reports that TNFα depolarizes skeletal muscle *in vitro*,[15] and that PAF causes membrane depolarization *in vivo*,[16] we tested these factors in our assay. Whereas plasma from hemorrhaged rats depolarized rat WBCs, neither 0.07 μmol/L (micromolar) TNFα, 7 μmol/L PAF-18, or a mixture of 0.05 μmol/L TNFα plus 5 μmol/L PAF-18 depolarized rat WBCs (Fig. 3). These same substances as well as heparin (tested because it was used in the collection of whole blood) did not depolarize

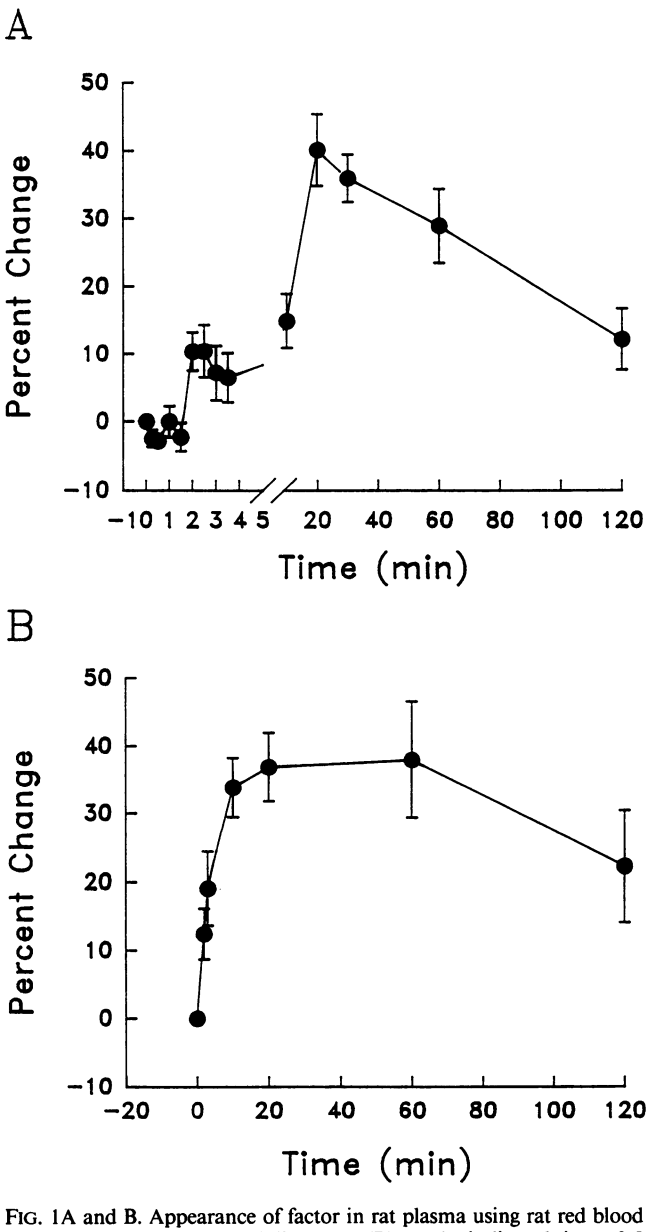

FIG. 1A and B. Appearance of factor in rat plasma using rat red blood cells (A) and the cultured cell line, a7r5 (B). At the indicated times, 0.5 mL of blood was drawn from cannulated rats and plasma was prepared as given in Methods. Red blood cells were diluted such that in the absence of plasma, when ultimately diluted to 3.0 mL for fluorometric determination, 50 μL would yield a fluorescence intensity of 25,000 to 35,000 cps or about 10%–15% of the maximum experimentally observed fluorescence amplitude. The remainder of the procedure is given in Methods. The ordinate is expressed as a percentage change relative to the intensity produced by incubation with plasma drawn in the first 0.5 mL of blood. Symbols indicate means and standard errors (n = 13).

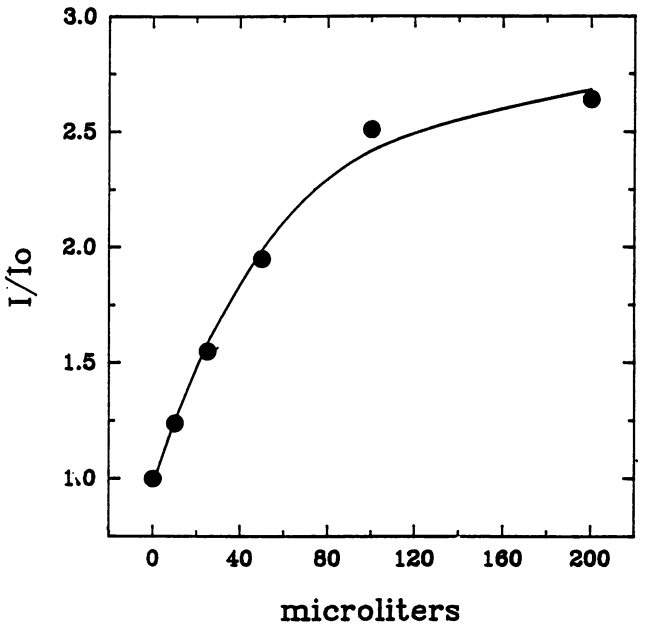

FIG. 2. Titration of RBCs with rat plasma Rat. Red blood cells (50 μL), the indicated volumes of plasma (drawn 60 minutes after hemorrhage), and buffer sufficient to bring the total to 400 μL were mixed and incubated for 15 minutes, then were washed as given in Methods. Symbols are averages of four determinations.

rat RBCs (Table 3). To eliminate the possibility that some plasma factor might be required for TNFα activity to become manifest, we incubated rat A-10 smooth muscle cells with control plasma and concentrations of TNFα ranging from 50 ng to 50 pg/mL[15] without observing depolarization (Fig. 4). Finally, to identify any contribution of TNFα to cellular depolarization, we incubated shock plasma with monoclonal anti-TNFα antibody (provided by Dr. Kevin J. Tracey, Cornell University Medical College) sufficient to yield a 100-fold molar excess, assuming a plasma concentration of 50 ng/mL TNFα. We observed no blockade of depolarization (Fig. 5). The report that TNFα depolarized excised rat muscle *in vitro*[15] suggested

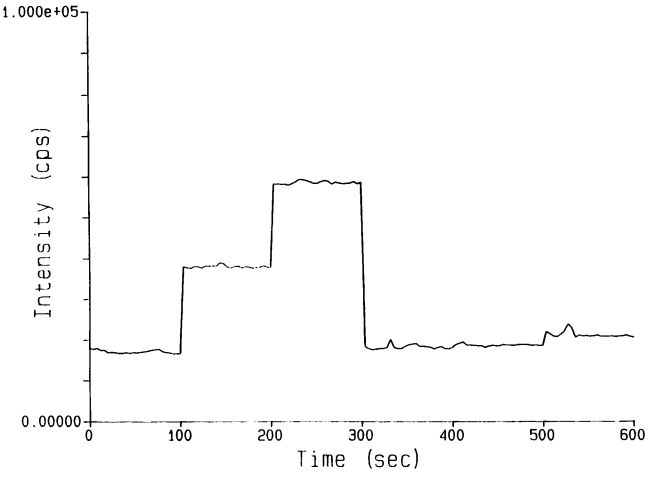

FIG. 3. Effect of TNF and PAF on rat WBCs. Rat WBCs were incubated as given in Methods and separate cuvettes were observed for 100 seconds starting at the indicated times. 0 Sec: Na/K buffer; 100 Sec: control plasma; 200 Sec: shock plasma; 300 Sec: 0.07 μmol/L human TNFα; 400 Sec: 7 μmol/L PAF; 500 Sec: 0.07 μmol/L TNF + 7 μmol/L PAF.

TABLE 3. *Effect of TNF and Heparin on Dog RBCS*

Incubation	Intensity	Intensity
Buffer	30,700	25,700
TNF	26,400	25,000
Heparin	29,300	25,000

Tumor necrosis factor (TNF) 5000 units (0.035 μmol/L [micromolar]); heparin 40 units (200 units/mL).

the possibility that TNFα might stimulate the release of endothelin from the blood vessels contained within the preparation. Therefore we tested endothelins 1, 2, and 3. None of the endothelins (0.1 μmol/L) depolarized rat RBCs (Table 4); however they had varying effects on the smooth muscle lines. Endothelin 3 but not 1 or 2 depolarized human intestinal smooth muscle (HISM), whereas endothelins 1 and 2 but not 3 depolarized rat A-10 aortic smooth muscle (Table 4).

Discussion

Shires and associates[1] identified depolarization of cellular membranes as the primary initial change in hemorrhagic shock. This inference was made from analysis of intracellular microelectrode measurements *in vivo* that show that the resting membrane electrical potential of skeletal muscle increases from −90 mV in normal tissues to −70 mV in tissues in shocked animals. This defect was confirmed by tissue analysis, which demonstrated increased intracellular water content, sodium and chloride

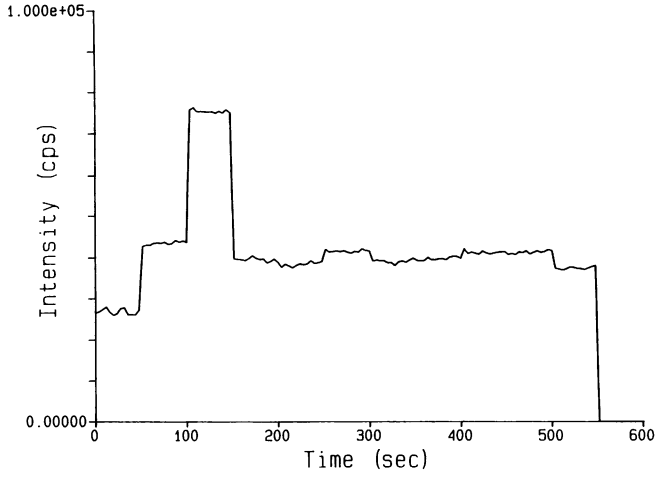

FIG. 4. Titration of A-10 cells with TNFα/control plasma. Rat aortic smooth muscle A-10 cells were incubated with control plasma and the indicated concentrations of TNF-α, washed, and fluorescence measured. Reaction mixtures were observed in separate cuvettes starting at the indicated times. All incubations with TNFα contained the same concentration of control plasma as did the sample assayed at 50 Sec. 0 Sec: buffer; 50 Sec: control plasma; 100 Sec: shock plasma; 150 Sec: 50 ng/mL; 200 Sec: 10 ng/mL; 250 Sec: 5 ng/ml; 300 Sec: 1 ng/mL; 350 Sec: 500 pg/mL; 400 Sec: 100 pg/mL; 450 Sec: 50 pg/mL; 500 Sec: 10 pg/mL.

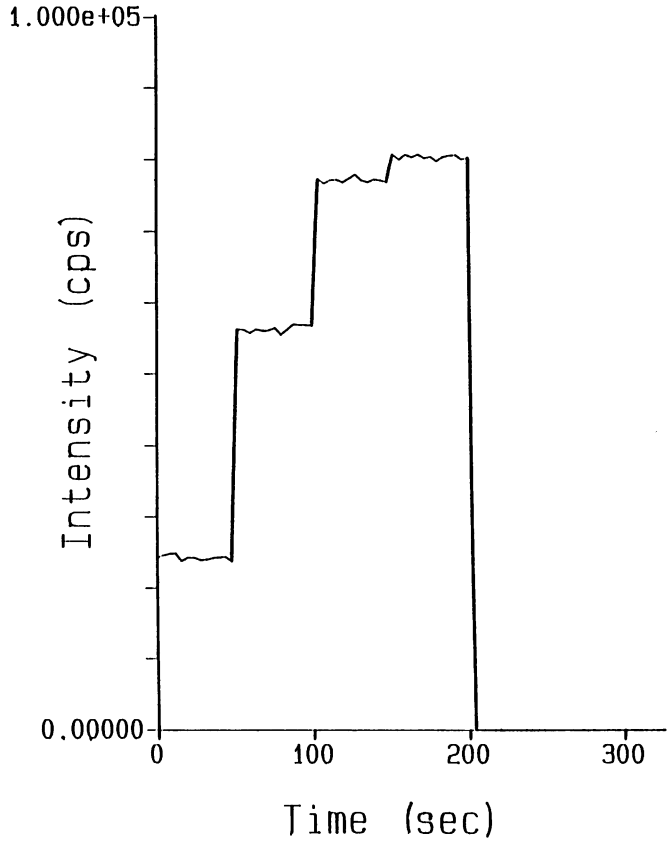

FIG. 5. Effect of anti-TNF-α antibodies on shock plasma. Monoclonal antibodies against TNFα was diluted in Na/K buffer such that 50 μL of the dilution would contain a 100-fold molar excess of antibody over TNF-α, assuming a plasma TNFα concentration of 50 ng/mL. Plasma was incubated with either antibody or an equivalent volume of buffer before incubation with H9C2 skeletal muscle cells. 0 Sec: buffer; 50 Sec: control plasma; 100 Sec: shock plasma; 150 Sec: shock plasma + anti-TNF-α antibody.

concentrations, as well as decreased intracellular potassium concentration.[17-19] Trunkey et al.[8] used microelectrodes to measure a 20-mV increase in the resting potential of skeletal muscle of a cross-perfused baboon, again suggesting a circulating factor. However this observation was not pursued further. Similar alterations in membrane functioning of RBCs were shown to accompany septic shock,[20] an observation that Gann[21] interpreted to imply the existence of a circulating factor responsible for the defect. The altered intracellular concentration of these ions suffices to account for the altered resting membrane potential as calculated by the Nernst equation.[22] Use of microelectrodes has been criticized because of several potential artifacts, including direct cellular injury resulting from puncture, unstable recording, leakage of ions around electrode, and leaching of ions from electrode.[14,23]

We desired an *in vitro* assay that would be sensitive to the presence of the presumptive depolarizing shock factor yet would not be subject to the same set of artifacts. Cer-

TABLE 4. *Effect of Endothelin*

A. Incubation	Incubation with Control Plasma: Intensity, Rat RBC	Incubation with Control Plasma: Intensity, HISM
Na/K buffer	41,000	61,400
Control plasma	120,000	87,100
Shock (1 hr)	132,900	100,000
CP + Endo 1	104,300	90,000
CP + Endo 2	102,900	88,600
CP + Endo 3	102,900	105,700

B. Endothelin	A-10 Smooth Muscle: Intensity(a)	Intensity(b)	Intensity(c)
Buffer	103,100	75,000	72,900
Endo 1	126,500	87,100	85,700
Endo 2	126,500	85,700	88,600
Endo 3	103,800	66,400	75,000

A: 0.1 μmol/L Endothelin (Endo) 1, 2 and 3. Control plasma (CP). B: Endothelin concentrations: (a), 1.0 μmol/L; (b): 0.1 μmol/L; (c): 0.01 μmol/L.

tain voltage-sensitive membrane dyes alter their absorbance or fluorescence linearly with changes in resting potential.[14,24,25] Fluorescence measurement of steady-state membrane potentials has been shown to be a sensitive and reproducible technique[14,26] that is not subject to the same artifacts that might limit the use of impaling microelectrodes. We chose to base our assays on the use of negatively charged oxonol dyes that tend to be excluded from the cell interior, which is itself negatively charged, rather than on positively charged dyes that tend to accumulate in the cells. Dyes that accumulate in the cell have greater toxic potential. In addition the more negatively charged subcellular membranous compartments tend to accumulate positive dyes differentially, complicating experimental interpretation.[23] To elucidate the biochemical mechanisms responsible for these physiologic phenomena, we developed an *in vitro* assay using cell-free plasma derived from hemorrhaged rats. We also required an assay with a high system throughout necessary for the many samples generated by biochemical kinetic and chromatographic procedures. However an *in vitro* assay would be useful only if there is a circulating factor that induces depolarization.

The lack of anatomic compartmentation and the metabolic simplicity of mammalian RBCs led many to question their usefulness as an experimental model of the physiology of more complex cells. However most of our knowledge of transmembrane electrolyte transport and consequent electric potential has been derived from work with RBCs.[27,28] In addition many benefits derive from the availability of RBCs in large numbers. Shires and collaborators[20] demonstrated a defect in sodium metabolism in RBCs in sepsis and in trauma patients[29] and in *in vivo* animal models.[8,19,30] This suggests that a common mechanism may underlie this phenomenon. In this report we show that *in vitro* fluorescence measurements may permit elucidation of the biochemical mechanisms mediating this defect.

We found that plasma obtained from rats after hemorrhage induced depolarization in various cell types relative to plasma obtained from rats not subject to hemorrhage. As noted above, similar depolarization was found in kidney cells regardless of whether they had undergone hemorrhagic shock. Thus conditioning of the cells during hemorrhage is not required.

To eliminate the possibility that the increased fluorescence resulted from the binding of dye to plasma proteins, we washed the cells after incubation with plasma. Nonspecific binding of dye to plasma proteins would result in an increase in fluorescence. Thus the observation that kidney cells incubated with control plasma and subsequently washed free of plasma had a lower intensity than similar cells incubated with buffer suggests that nonspecific protein binding does not account for these observations. Also because the medium was buffered to pH 7.4, changes in hydrogen ion concentration also cannot account for our observations. Except in the initial experiments, all data were obtained using naive cells, that is cells not taken from a hemorrhaged animal. The difference in response to shock *versus* control plasma of the many cell types tested implies the existence of a soluble factor(s) that is expressed differentially in the plasma of a hemorrhaged animal.

It is generally believed that in shock cellular hypoxia, usually resulting from ischemia, leads to depolarization of the cell membrane,[1] perhaps mediated by decreased production of adenosine triphosphate.[31,32] However the RBC depolarizes despite the fact that it does not depend on oxygen for metabolism. Furthermore the cells in the present study were not subjected to an experimental period of ischemia or hypoxia, so a limitation in oxygen availability to the target cell is not required for the development of depolarization. However ischemia may be required for the production of the circulating factor(s).

We sought additional evidence that the increase in fluorescence did not result from nonspecific effects of plasma proteins or ions. Figure 2 shows that the amplitude of the response initially increases with increasing dose of plasma and then saturates. Saturation is indicative of specific equilibrium receptor-binding processes. For these experiments, the normal 30-minute incubation was shortened to 15 minutes, following which the RBCs were washed free of plasma. Thus it would seem that RBCs react rapidly to plasma. For some experiments, the plasma was dialyzed overnight at 4°C *versus* Na/K buffer. Dialyzed shock plasma depolarized tested cells relative to similarly treated

control plasma. These results suggest that the effective principle(s) is not an ion or other low-molecular-weight (MW) substance; rather the 10,000 MW cut-off of the dialysis membrane suggests the factor is intrinsically of high MW, or an oligomer, or exists bound as a high MW complex. In preliminary experiments we did not take special precautions against proteolytic digestion. Under these conditions a given preparation of dialyzed plasma lost ability to depolarize unpredictably with time. The addition of 1 mmol/L ethylenediaminetetraacetic acid and aprotinin (200 KIU/mL) afforded more stable preparations. These results suggest that the factor(s) either is itself a protein or is bound to a carrier protein.

Tables 1 and 2 show that a wide range of mammalian species and cell types respond to rat shock plasma. In fact every cell type tested responded positively to shock plasma. However skeletal muscle responded only after having differentiated in culture (data not shown); this may indicate maturation-dependent acquisition of the appropriate receptor. Figure 1 suggest that the circulating factor(s) exists as a readily releasable pool with indications of possible further synthesis at later time.

Both *in vitro* application of TNFα[15] and *in vivo* application of PAF[16] induce membrane depolarization. Our results (Figs. 3 and 4) indicate that these effects do not result from the direct action of these agents on the target cells. Neither TNFα nor PAF alone or in combination, even at supraphysiologic concentrations, depolarized any of the cell types tested. Furthermore monoclonal antibodies to TNFα did not affect the depolarization induced by shock plasma (Fig. 5). Heparin also was ineffective in our hands. Because the excised muscle used by Tracy et al.[15] contained blood vessels, it is possible that TNFα acted by release of a substance such as the endothelins. Endothelins gave mixed results in the depolarization assay (Table 4). None of the endothelins tested was active against RBCs. However both endothelins 1 and 2, but not endothelin 3, depolarized aortic smooth muscle, whereas endothelin 3 depolarized HISM. Because endothelin did not depolarize all the cell types affected by shock plasma, we do not think it is the sole active principle in hemorrhagic shock. Therefore we did not pursue this observation in greater detail in this study. However, if striated muscle reacts as does smooth muscle, one or more endothelins might be the final principle active in the report of Tracey et al.[15]

The observed membrane defect is associated with an increased intracellular sodium concentration.[22] To ascertain the requirement for exogenous sodium, we incubated RBCs and shock plasma either in a high sodium buffer or in a high potassium low sodium buffer. We observed that dog and pig RBCs were depolarized by rat shock plasma only in the presence of high sodium buffers (Table 1). This observation is consistent with the hypothesis that a defect in the export of cellular sodium combined with passive influx of extracellular sodium is responsible for the observed depolarization.[28]

Our results indicate a 20% to 30% increase in DIBAC fluorescence after incubating muscle cells with shock plasma. This would correspond to a 20- to 30-mV increase in the resting membrane potential, which correlates with the observations of Shires and associates.[1,8] This correlation supports our ability to use this assay to elucidate the biochemistry of hemorrhagic shock. We are using the techniques developed in this report to monitor chromatographic purification of the active principle.

Acknowledgments

The authors thank John Williams for technical assistance.

References

1. Chiao JJC, Minei JP, Shires III GT, Shires GT. *In vivo* myocyte sodium activity and concentration during hemorrhagic shock. Am J Physiol 199; 258 (Regulatory Integrative Comp Physiol 27):R684–R689.
2. Ravin HA, Schweinberg FB, Fine J. Host resistance in hemorrhagic shock: IX. Isolation of toxic factor from hemorrhagic shock plasma. Proceedings of Society of Experimental Biology and Medicine 1958; 99:426–431.
3. Blattenberg B, Levy MN. Humoral reticuloendothelial-depressing substance in shock. Am J Physiol 1962; 203:111–113.
4. Fukuda T. Endogenous shock-inducing factor in caninehemorrhagic shock. Nature 1969; 205:392–393.
5. Nagler AL, Levenson SM. Presence of a passively transferable lethal factor in the blood of rats in severe hemorrhagic shock. J Trauma 1972; 12:608–612.
6. Lefer AM. Blood-borne humoral factors in the pathophysiology of circulatory shock. Circ Res 1973; 32:129–139.
7. Arango A, Illner H, Shires GT. Role of ischaemia in the induction of changes in cell membrane during hemorrhagic shock. J Surg Res 1976; 20:473–476.
8. Trunkey DD, Illner H, Arango A, Holliday R, Shires GT. Changes in cell membrane function following shock and cross perfusion. Surgical Forum 1974; XXV:1.
9. Beauge L. Inhibition of translocation reactions by vanadate. Methods in Enzymology 1988; 156:251–267.
10. Engeland WC, Miller P, Gann DS. Dissociation between changes in plasma bioactive and immunoreactive adrenocorticotropin after hemorrhage in awake dogs. Endocrinology 1989; 124:2978–2985.
11. Darlington DN, Keil LC, Dallman MF. Potentiation of hormonal response to hemorrhage and fasting, but not hypoglycemia in conscious adrenalectomized rats. Endocrinology 1989; 125:1398–1406.
12. Raff H, Fagin KD. Measurement of hormones and blood gasses during hypoxia in conscious cannulated rats. J Appl Physiol Respir Environ Exercise Physiol 1984; 56:1426.
13. George EB, Nyirjesy P, Basson M, Ernst LA, Pratap PR, Freedman JC, Waggoner AS. Impermeant potential-sensitive oxonol dyes: I. Evidence for an "on-off" mechanism. J Membr Biol 1988; 103: 245–253.
14. Brauner T, Hulser DF, Strasser RJ. Comparative measurements of membrane potentials with microelectrodes and voltage-sensitive dyes. Biochimica Biophysica Acta 1984; 771:208–216.
15. Tracey KJ, Lowry SF, Beutler B, et al. Cachectin/Tumor Necrosis Factor mediates changes of skeletal muscle plasma membrane potential. J Exptl Med 1986; 164:1368–1373.

16. Minei JP, Shires GT III, Jones DB, Shires GT. Platelet activating factor antagonist CV3988 prevents hepatic cellular membrane dysfunction during live E. coli bacteremia. Surg Forum 1988; 39:24–26.
17. Nakayama S, Kramer GC, Carlsen RC, Holcroft JW. Infusion of very hypertonic saline to bled rats: membrane potentials and fluid shifts. J Surg Res 1985; 38:180–186.
18. Pietzman AB, Corbett WA, Shires GT III, et al. Cellular function in liver and muscle during hemorrhagic shock in primates. J Clin Surg 1982; 1:315–322.
19. Shires GT, Cunningham JN, Baker CRF, et al. Alterations in Cellular membrane function during hemorrhagic shock in primates. Annals of Surg 1972; 176:288–295.
20. Shires GT III, Peitzman AB, Illner H, Shires GT. Changes in red blood cell transmembrane potential, electrolytes, and energy content in septic shock. J Trauma 1983; 23:769–774.
21. Gann DS. Discussion of reference 19 above, 1983.
22. Shires GT, Shires III GT. Cellular Response. *In* Shires GT, ed. Shock and Related Problems. New York: Churchill Livingstone, 1985, pp 15–24.
23. Grinstein S, Dixon SJ. Ion transport, membrane potential, and cytoplasmic pH in lymphocytes: changes during activation. Physiol Rev 1989; 69:417–481.
24. Cohen LB, Salzberg BM. Optical measurement of membrane potential. Rev Physiol Biochem Pharmacol 1978; 83:35–88.
25. Waggoner AS. Dye indicators of membrane potential. Ann Rev Biophys Bioeng 1979; 8:47–68.
26. Wilson HA, Chused TM. Lymphocyte membrane potential and Ca2+ sensitive potassium channels described by oxonol dye fluorescence measurements. J Cellu Physiol 1985; 125:72–81.
27. Tosteson DC, Shea E, Darling RC. Potassium and sodium of red blood cells in sickle cell anemia. J Clin Invest 1952; 31:406–411.
28. Illner H. Changes in red blood cell transport in shock. *In* Shires GT, ed. Shock and Related Problems. New York: Churchill Livingstone, 1985, pp 25–43.
29. Cunningham JN, Shires GT, Wagner Y. Changes in intracellular sodium and potassium content of red blood cells in trauma and shock. Am J Surg 1971; 122:650–654.
30. Holliday RL, Illner H, Wagner YI, Shires GT. Liver cell membrane alterations during hemorrhagic shock in the rat. J. Surg Res 1981; 31:506–515.
31. Baue AE, Chaudry IH, Wurth MA, Sayeed MM. Cellular alterations with shock and ischemia. Angiology 1974; 25:31–42.
32. Chaudry IH, Clemens MG, Baue AE. Alterations in cell function with ischemia and shock and their correction. Archives of Surgery 1981; 116:1309–1317.

Discussions

Dr. Basil Pruitt (San Antonio, Texas): Dr. Gann and his colleagues have presented data suggesting that a circulating toxin capable of impairing cell function is induced by hemorrhagic shock. These data supporting the earlier findings of Dr. Shires and others suggest that a humoral factor that is neither TNF nor PAF appears in the plasma within 5 minutes of shock and causes the depolarization of a wide variety of cells.

That the factor remains uncharacterized raises the possibility that it is the Phoenix of myocardial depressant factor and burn toxin, the effects of which have been attributed to salt concentration and endotoxin, respectively.

In these studies the possibility also exists, unless precautions were taken to exclude endotoxin, that this factor actually may be endotoxin absorbed from the gut or released from the liver. The dialysis procedure described does not exclude endotoxin because endotoxins are a family of substances that vary in molecular size from 10,000 to near 2.4 million daltons because of the tendency of lipid A to form aggregates.

Even though the technology used in these studies does not necessitate impalement and, therefore, injury to the cells, there are variables that influence the fluorescent intensity of oxonol dyes. The voltage-sensitive fluorescent dyes such as oxonol bind to proteins.

Because that is true, the fluorescence of the test system would increase if the protein content of the shock plasma were greater than that of the control plasma.

Similarly fluorescence is influenced by cell size and decreases as the membrane surface area per unit cell volume is increased. If exposure to shock plasma causes the volume of the cells in the test system to decrease, measured fluorescent intensity would increase, independent of other factors.

In addition the authors note that the measurement of decreased potential difference was dependent on the sodium content of the test system. Other investigators using oxonol 6 to measure membrane potentials found that as sodium concentration decreases, the transport mode of the sodium potassium ATPase pump mechanism changes from electrogenic to electroneural. And I ask whether the sodium concentration in either the plasma or in the cells in the test system varied between the control and the shock samples.

Recent studies of inside-out vesicles from red blood cells have shown that membrane potentials decrease as pH is increased. I would ask the author whether the pH of the shock plasma systematically differed from that of the control plasma.

I compliment the authors on their application of new noninvasive technology to the pathophysiology of shock. They note that preliminary studies are consistent with the factor being a protein or bound to a protein. And that raises the possibility that an antibody can be developed that will counteract the effects of the factor and reverse the cellular impairment characteristic of early shock.

Dr. James Carrico (Dallas, Texas): After Dr. Pruitt's eloquent and detailed discussion, my job is fairly simple. I would like to emphasize the potential importance of this study, raise a note of caution, and then ask Dr. Gann two questions.

We have been told that there is activity in serum taken from animals subjected to shock that produces depolarization of a number of cells *in vitro*.

The importance of this study is multiple, but among the potential importance is that it validates the observations of Dr. Shires and his coworkers by totally different methodology.

It explains a number of observations that have been hanging, waiting explanation, including the difficulty of tourniquet-induced ischemia to produce similar depolarization and including some cross-circulation observations published by Trunkey when he was working in Dr. Shires' lab.

As Dr. Pruitt mentioned, it opens new avenues for understanding the pathophysiology of shock and potentially new therapeutic modalities. So the potential importance is there.

The caution is also raised by Dr. Pruitt, and that can be phrased as: Have we been here before? The myocardial depressant factor in various names and various reports has, again and again, raised our enthusiasm only to be discovered to be related to some methodologic quirk.

So the obvious question and the obvious caution is to carefully look at the methodology and determine if there some is methodologic quirk that is producing these findings.

What Dr. Gann has really showed us is that there is activity in shock serum that increases the fluorescence of cells treated with a super vital dye that is, among other things, responsive to changes in membrane potential.

Dr. Pruitt mentioned a number of other factors that can change the fluorescence of such dyes, and those need to be investigated.

My questions are very general and fairly simple. Can you give us any more infirmation about what you have done to rule out some of the other causes of alteration in fluorescence: changes in iron concentration, changes in protein concentration, changes in cell volume, changes in amount of dye attached to the cell, and changes in the nature of the fatty acids of the cell membrane itself?

Have you validated this observation by other methods; for instance administration of this serum to other animals and observation of not fluorescence of red cell membranes but rather intracellular sodium content of red cells, which would suggest this same change?