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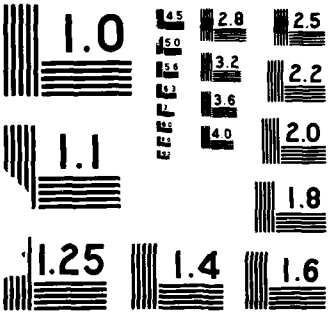
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ABSTRACT

Platelet aggregation in response to adenosine diphosphate (ADP), epinephrine, collagen, thrombin, ristocetin and phorbol myristate acetate (PMA) was studied at 1 ATA (air) and 35 ATA (helium) as was platelet shape change. Pressure inhibited aggregation in response to all agents except PMA and it had no effect on shape change. Dose response curves were constructed for ADP and epinephrine at 1 ATA and 35 ATA in the presence and absence of acetylsalicylic acid (ASA) 2.5×10^{-4} M (.00025) final concentration. With primary aggregation thus isolated, smoother dose responses were obtained and pressure flattened the response in the higher dose range, a behavior suggestive of non-competitive blockade or reduced availability of receptors. All of the agents that were inhibited by pressure are dependent upon extracellular Ca^{2+} for their function. All unmask other receptors (integrins) for adhesive proteins, principally fibrinogen. These integrins incorporate Ca^{2+} to become active. In contrast, PMA aggregation and shape change both are independent of extracellular Ca^{2+} (EDTA was used as a chelator of Ca^{2+}) and were unaffected by pressure. It is proposed that pressure distorts Ca^{2+}-dependent surface glycoprotein receptors in a manner that reduces ligand affinity and hence inhibits platelet aggregation. (A-1)

ONR PROGRAM ON MACROMOLECULAR AND CELLULAR EFFECTS OF PRESSURE

ANNUAL REPORT, grant N 00014-88-J-1108

Effects of Pressure and Anesthetics on Cell Membranes and Secretory Processes

Objectives:

The general objective of this project is to improve our understanding of the effects of both pressure and anesthetics (including inert, narcotic gases) on cell function, particularly the cell membrane, and to determine whether, in fact, the opposing actions of pressure and anesthetics are exerted through a common site. It is hoped that useful information also will be acquired that may shed light on why centrally-acting drugs are affected in an apparently-unpredictable fashion by high pressures of inert gases. Specifically, the following questions will be addressed:

1. Are different surface receptors for ligands affected differently, qualitatively or quantitatively, by hyperbaric conditions?

2. Are membrane-associated events that are not receptor-mediated similarly affected?

3. Are internal regulatory processes also altered and, if so, how?

4. Can an isolated protein or artificial membrane be shown to respond to hyperbaric conditions in a manner that might indicate the relative importance of protein and lipid components?

Progress in 1988-89:

Approval for this project was obtained in May of 1988 but funding was not available until August 1. Since that time work has centered on the use of the human platelet as a model for the study of cell membrane events. Platelet aggregation was studied over a range of pressures up to 35 ATA using as agonists adenosine diphosphate (ADP), epinephrine, thrombin, collagen, ristocetin and phorbol myristate acetate (PMA). Initially, dose responses were constructed for pressures of 1, 12, 25 and 35 ATA of helium (He) and nitrogen N2) using a fixed concentration of ADP or epinephrine. Both gases were inhibitory in a pressure-dependent fashion with N2 being more potent than He (which is taken to represent hydrostatic pressure).

Dose response curves were then constructed for ADP and epinephrine at 35 ATA of He in order to determine the effects of pressure per se on a ligand-receptor event. With both agonists, the dose response curves were shifted to the right, indicating significant inhibition by pressure. However, aggregation induced by the agents is accompanied by the secretion of platelet granular constituents (ADP, serotonin, thrombin) and by the formation of arachidonic acid and thromboxane A2, all of which promote aggregation, thus confusing the interpretation of

the results. The study of ADP and epinephrine was therefore repeated but in the presence of 10-4 M acetylsalicylic acid (ASA), an inhibitor of cyclooxygenase and hence of the secretory response to these agonists. Once again, the dose response curves were shifted to the right, indicating significant inhibition by pressure. In this case, the curves were smoother, with smaller standard deviations, as a result of the removal of the influence of secreted, pro-aggregatory agents.

When the reciprocals of equipotent concentrations of an agonist in the presence and absence of an antagonist (in this case 35 ATA pressure) are plotted against each other and the regression calculated, some information can be obtained regarding the nature of the inhibition. When this was done for ADP and epinephrine, neither regression line passed through the origin. This is taken to indicate that an increase in the concentration of the agonist cannot completely overcome the inhibitory influence of the antagonist. When the antagonist is a drug, this indicates a noncompetitive antagonism or a "hemiequilibrium state" as defined by Paton and Waud (J. Physiol. Lond. 191:59, 1967). Since in this case the antagonist is pressure, the only plausable explanation is that a change in ligand-receptor affinity has occurred or that some downstream event regulating cell function has been altered in a noncompetitive manner.

Statistically-significant inhibition of platelet aggregation by 35 ATA of He was observed when ADP, epinephrine, collagen, ristocetin or thrombin (at two concentrations) were the aggregation stimuli. The exception was PMA, which enters the cell to directly stimulate protein kinase C (thus acting as a substitute for diacyl glycerol (DAG)) and which does not require the participation of extracellular calcium. ADP, epinephrine, collagen and ristocetin all do, and thrombin appears to work through both calcium-dependent and -independent pathways.

To further investigate the role of calcium, shape change in response to ADP, collagen and ristocetin was studied in the presence of calcium chelation by disodium EDTA. Shape change is reflected in an increase in optical density of the platelet suspension caused by a shift from the quiescent discoid form to the pre-aggregation spheroidal form. It is accompanied by centralization of the granules and the formation of myosin strands and it requires the formation of ITP and DAG and the mobilization of internal calcium stores. Most signifacantly, it does not require the presence of extra-cellular calcium (Steen and Holmsen,Eur J Haematol 38:383, 1987). Shape change in response to ADP, collagen and ristocetin, as measured by the increase in optical density, was not affected by a pressure of 35 ATA. Shape change in response to a low dose of thrombin also was not affected by this pressure. At a higher dose, aggregation was

initiated. These results indicate that pressure modifies some process that is dependent on extracellular calcium. One possible site is a class of surface receptor known as integrins. These surface glycoproteins appear to be involved in all cell-cell adhesive reactions by forming bridges with fibrinogen, von Willebrand's factor, lectins and other adhesive proteins. This process requires the participation of extracellular calcium (Ruoslahti and Piersbacher, Science,238: 491, 1987).

Integrin receptors are normally in a nonreceptive configuration and are unmasked in response to ligands such as ADP. This may occur directly , without the participation of transducing G proteins , or indirectly through such proteins by the activation of protein kinase C and the formation of ITP and mobilization of intracellular calcium (Gerrard,Hosp Practice, 23: 89, 1988). Since these latter pathways are also involved in shape change, which was unaffected by pressure, it seems likely that the direct activation of surface integrins is inhibited by pressure.

These results formed the basis of a manuscript that was submitted recently to "Undersea Biomedical Research."

Pilot Studies:

A liason has been established with Dr. Patrick T.T. Wong of the division of Chemistry, National Research Council Laboratories in Ottawa. Dr. Wong is a physical chemisist expert in the effects of very high (kilobar range) pressures on molecular interactions. He also is interested in the biological application of such research, but feels deficient in his knowledge of this field. We have conducted some preliminary experiments with an artificial model membrane and the results indicate that pressure influences the interaction between a ligand and its protein binding site. Over the next year we intend to refine this technique, which employs Fourier transform infrared spectroscopy (Wong et al, Appl. Spectros. 39:733, 1985) and to attempt to show similar effects on ligand-receptor interactions at more physiologically-relevant pressures.

Preliminary studies have been done on the effects of nitrous oxide (N2O) and xenon (Xe) on platelet aggregation. Predictably, N2O inhibited aggregation in a concentration (pressure) dependent manner. Suprisingly, Xe had little effect on aggregation even at 3.5 ATA, despite its almost equal anesthetic potency with N2O. These results need to be confirmed and if they are, the basis of the difference established, as it could shed light on underlying mechanisms of anesthesia.

We have also begun to investigate the trans-membrane movement of calcium into platelets in response to agonists such as ADP using fluorescent detection of calcium-binding dyes such as fura-2 (Sage and Rink,J. Biol. Chem.,262: 1634, 1987). The

effects of pressure on calcium movement in this and other cell models will be studied provided that suitable pressure chambers can be designed to fit the instrumentation. A hyperbaric engineering firm, Fullerton- Sherwood of Mississauga Ont. has been consulted to advise and submit a design.

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Program on Macromolecular and Cellular Effects of Pressure

INVESTIGATORS

Dr. Rodney L. Biltonen
Dept. of Pharmacology
University of Virginia
Charlottesville, VA 22908

Dr. P. A. George Fortes
Department of Biology, C-016
University of California
La Jolla, CA 92093

Dr. Hans Frauenfelder
Loomis Laboratory
University of Illinois
1110 West Green Street
Urbana, IL 61801

Dr. Perry M. Hogan
Department of Physiology
State University of New York
Buffalo, NY 14214

Dr. Thomas F. Murray
College of Pharmacy
Oregon State University
Corvallis, OR 97331-3507

Dr. Richard B. Philp
Dept. of Pharmacology & Toxicology
The Univ. of Western Ontario
Medical Sciences Building
London, Ontario, Canada N6A 5C1

Dr. Shalom R. Rackovsky
Department of Biophysics
University of Rochester
School of Medicine and Dentistry
602 Elmwood Avenue
Rochester, NY 14642

Dr. Lou Reinisch
Laser Biophysics Center
F. Edward Hebert School
of Medicine
4301 Jones Bridge Road
Bethesda, MD 20814-4799

Dr. Joseph F. Siebenaller
Dept. of Zoology and
Physiology
Louisiana State University
Baton Rouge, LA 70803-1725

Dr. Anne Walter
Dept. of Physiology and
Biophysics
Wright State University
Dayton, OH 45435

Dr. William B. Weglicki
Division of Experimental
Medicine
Ross Hall, Room 409
2300 Eye Street, N.W.
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