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Dr. Peter Kent
Department of the Navy
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NMRDC-40
Naval Medical Command, National Capitol Region
Bethesda, MD 20814-5044

Dear Dr. Kent:

Pleas find attached the final report for project MM33P30/Task 4 -- Diving biotechnology entitled "Amino acid neurotransmitters and high pressure nervous syndrome". I have also enclosed a preprint of our most recent paper that should appear soon in *Glia*.

Sincerely,

David L. Martin, Ph.D.
Chief, Laboratory of Neurotoxicology
and Nervous System Disorders

Final Report for Project MM33P30/Task 4 -- Diving Biotechnology

Title: Amino acid neurotransmitters and high pressure nervous syndrome.

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Nontechnical summary:

High pressure nervous syndrome (HPNS) occurs in humans and animals exposed to high atmospheric pressures such as those experienced during deep diving. The primary symptoms of HPNS, which are tremors and seizures, may result from excessive excitatory or inhibitory neurotransmission in the central nervous system. This project comprised two components. The first was a detailed study of the mechanisms by which excitatory and inhibitory transmitters are released from astroglial cells *in vitro* and the effects of high pressure on these processes. The second component was the analysis of amino acids (specifically including glutamate, aspartate, taurine, and γ-aminobutyric acid) of perfusion fluids and tissue extracts received from the Hyperbaric Medicine Program Center, NMRI, Bethesda, MD.

The results of our studies showed that the release of neuroactive compounds by astroglia (specifically taurine and the adenine-labelled compounds cyclicAMP and ATP) is highly sensitive to changes in the osmolality of the medium and cell volume (changes in osmolality as small as 2.5% significantly effect taurine release) but is relatively insensitive to changes in hydrostatic pressure. The high osmotic sensitivity of these release processes release appears to be physiologically significant and may play a role in the physiological responses to circumstances that result in changes in blood osmolarity, such as dehydration, that are encountered by armed services personnel. On the other hand, the low sensitivity of these release processes to hydrostatic pressure suggests that the hydrostatic pressure does not induce HPNS by acting directly on astrocytes *per se* and changing the release of taurine and adenine-labeled compounds. However, because the release of taurine and adenine-labeled compounds appears to be altered by small physical distortions of the astrocytes, it is possible that high-pressure induced phsyical changes could indirectly modify astrocytic release processes and thereby be in HPNS.

Experimental findings:

Release of neuroactive amino acids and adenine-labeled compounds from astroglial cells:

Astroglial cells release taurine and adenine-labeled compounds when stimulated with β-adrenergic agonists such as isoproterenol (IPR) and elevated $[K^+]_o$. Our release studies were carried out astrocytes in primary culture and with LRM55 cells, a continuous cell line with many astrocytic properties. Our goals were to characterize the release

mechanism(s) and determine the effects of elevated pressure on them. Our results (see below) showed that the release processes are highly sensitive to transmembrane differences in osmotic pressure but insensitive to changes in hydrostatic pressure. Our results have lead us to hypothesize that increased membrane tension, such as induced by cellular swelling, leads to taurine release and suppresses the release of adenine-labeled compounds.

As described in the articles listed below, we found that the mechanism responsible for taurine release from astroglia differs from that responsible for transmitter release from neurons because (a) β-agonists do not depolarize astrocytes (C. Bowman, personal communication), (b) β-agonists do not induce changes in $[Ca^{2+}]_i$, (c) changing $[Ca^{2+}]_i$ does not induce release, (d) release is independent of $[Ca^{2+}]_o$, and (e) taurine is released from the cytoplasmic pool not a distinct releasable pool.

It is sometimes assumed that K^+-stimulated release results from K^+-induced depolarization, as in neurons where depolarization leads to Ca^{2+} influx and the activation of vesicular release, but our studies indicate that K^+-stimulated taurine release from astrocytes involves more than simple depolarization. First, elevated $[K^+]_o$ stimulates taurine release from LRM55 astroglial cells at low concentrations (10-20 mM) that have little effect on membrane potential. Furthermore elevated $[K^+]_o$ only stimulates taurine release from astrocytes and LRM55 cells when $[K^+]_o$ is raised isosmotically (e.g. by substituting KCl for NaCl) and does not stimulate release when $[K^+]_o$ is raised hyperosmotically by adding KCl to the medium. In the latter case, even high, strongly *depolarizing* $[K^+]_o$ (e.g. 50 mM) does not stimulate release. K^+-stimulated release is highly sensitive to the osmolality of the medium; release is suppressed by small increases in osmolality (2.5-10%) and enhanced by similar small decreases in osmolality. Even though depolarization is immediate, K^+-induced release increases slowly over a period of 10-15 min, a time course similar to KCl uptake in high-K^+ medium. The slow time course, the osmotic sensitivity, and failure of depolarization *per se* to induce release led us to hypothesize that K^+-stimulated taurine release is secondary to swelling induced by KCl uptake. In accordance with this idea, we found that K^+-stimulated release was highly Cl^- dependent. For example, taurine release induced by 50-mM $[K^+]_o$ was virtually eliminated when $[Cl^-]_o$ was reduced to 12 mM, a concentration chosen to keep the $[K^+][Cl^-]$ product equal to that in control medium, thereby minimizing the ion shifts and swelling resulting from Donnan forces.

We have found that high-K^+-stimulated ATP release has the opposite osmotic sensitivity -- increases in osmolality increase release and decreases in osmolality reduce release. These results, along with data showing that taurine is selectively released in comparison to other amino acids, indicates that taurine release is not simply a non-specific response to swelling.

Although β-agonist-stimulated taurine release is a cAMP-mediated process, it also is exquisitely sensitive to the osmolality of the medium; it is almost completely suppressed

when the osmolality is raised by 15 mosM (ca. 5%) with sucrose or NaCl and is greatly enhanced when the osmolality is reduced by 2.5-10%. Furthermore β-agonist-stimulated release is greatly enhanced when the β-agonist is applied at the peak of K^+-stimulated release. Thus, the release response to β-agonists and elevated $[K^+]_o$ have a clear commonality in their osmotic sensitivity. Each of these compounds also induces changes in cell dimensions -- elevated $[K^+]_o$ induces cell swelling and β-agonists induce a rearrangement of cytoskeletal proteins and a withdrawal of cytoplasm and cell membrane causing the cell to change from a flat polygonal morphology to a stellate shape.

Thus, we hypothesized that any factor that induces a change in cell dimensions can affect taurine release. In particular, if a factor increases the tension between the cytoskeleton and the taurine release mechanism in the cell membrane, taurine release will be increased. Conversely, a decrease in tension will suppress release. Thus, K^+-induced cell swelling and β-agonist-induced retraction of the cytoplasm and cell membrane will each increase release. Furthermore, they would be expected to have additive or synergistic effects, since each will add to the tension caused by the other. On the other hand, shrinking the cells with hyperosmotic medium would be expected to inhibit β-agonist-induced release since it would take greater movement to generate the tension necessary to induce release.

We have developed an approach to identify the molecular mechanism responsible for release by examining changes in protein phosphorylation during IPR-stimulated release. IPR stimulates cAMP synthesis which than activates a protein kinase that transfers a phosphate from ATP to the appropriate protein substrate. Cells are incubated with $^{32}PO_4$ and allowed to synthesize γ-^{32}P-ATP. The cells are then stimulated with IPR causing the phosphorylation of proteins. The proteins are extracted and analyzed by 2-dimensional gel electrophoresis and autoradiography. Phosphoproteins are identified as densities on the autoradiograms. The autoradiograms are analyzed by using a sophisticated computer program and data base. Using this approach we can routinely identify more than 75 phosphoproteins in control cells. Comparison of control cells with IPR-stimulated cells shows that phosphorylation of some proteins is increased while phosphorylation of others is decreased. Comparison of the time course of labeling with the time course of release suggests that one of a small subset of labeled proteins is involved in release.

Effects of elevated hydrostatic pressure on the release of taurine and adenine labeled compounds:

Although osmotic pressure differences had strong effects on the release of taurine and adenine labeled compounds from astroglial cells, elevated hydrostatic pressure had little or no effect.

Studies of the effects of pressure on taurine release from astroglial cells required the development of a new apparatus for superfusing the cells. A stainless steel perfusion system proved unsatisfactory, apparently because the cells were poisoned by small

amounts of Fe and Cr ions leached from the steel. An apparatus that could generate up to 5 MPa (750 psi) was constructed of glass and plastic and proved to be satisfactory. IPR-stimulated taurine release was slightly reduced (*ca.* 20%) by the application of high pressure (4 MPa) but elevated pressure had no discernable effect on release evoked by 50mM $[K^+]_o$ (release under high pressure = 105 ± 11 % of control, n = 6).

The effect of pressure on K^+- and IPR-stimulated release of adenine-labeled compounds (cAMP and ATP) was investigated as a control for taurine release, since these compounds are released by a different mechanism than taurine and might therefore respond differently to elevated pressure. Elevated pressure (3.94 ± 0.06 MPa) did not affect the release of release of adenine derivatives whether stimulated by high $[K^+]_o$ (release under high pressure = 108 ± 15 % of control, n = 6) or by IPR (100 ± 4% of control).

Analysis of amino acids in tissue extracts and superfusates:

The analysis of tissue extracts and superfusion fluids required the implementation of improved methods of analysis due to the low concentrations of amino acids in the superfusion fluids. Initial experiments showed that superfusion medium that had never been exposed to tissue contained high enough levels of amino acids to interfere with the analysis. The levels of interfering amino acids was reduced by using higher purity reagents and better methods of preparing the superfusion media. The analytical method itself was improved by incorporating the use of an internal standard and by developing a "sandwich technique" for the automatic injection system to increase the size of the sample that could be analyzed by eight fold. In this method the sample was loaded in between to injections of derivatizing solution.

A substantial number of samples (338) were analyzed for the Hyperbaric Medicine Program Center, NMRI, Bethesda, MD. Preliminary review of these results indicates that high pressure causes a delay in the release of GABA and glutamate from nerve endings, and that elevated $[K^+]_o$ does not stimulated the release of taurine from nerve endings.

Publications:

We continue to publish the work done in this area. Seven articles and one book have appeared. Three additional articles are in preparation at this time and will be submitted as supporting documents after they are accepted for publication.

Articles:

Shain, W. Connor, J.A., Madelian, V. and Martin, D. L. Spontaneous and Beta-adrenergic Receptor-mediated Taurine Release from Astroglial Cells Are Independent of Intracellular Calcium. J. Neurosci. 9, 2306-2312 (1989).

Martin, D. L., Madelian, V. and Shain, W. Spontaneous and Beta-adrenergic Receptor-mediated Taurine Release from Astroglial Cells Do Not Require Extracellular

Calcium. J. Neurosci. Res. 23, 191-197 (1989).

Martin, D. L., Madelian, V., Seligmann, B. and Shain, W. The role of osmotic pressure and membrane potential in K^+-stimulated taurine release from cultured astrocytes and LRM55 cells. J. Neurosci. 10, 571-577 (1990).

Shain, W., and Martin, D,L. (1990) "Taurine uptake and release -- an overview" in Taurine: Functional Neurochemistry, Physiology and Cardiology" pp 243-252.

Shain, W. Madelian, V. Waniewski, R. and Martin, D.L. (1990) "Characteristics of taurine release from astroglial cells" ibid. 299-306.

Martin,D.L. Madelian, V., and Shain, W. (1990) "Osmotic sensitivity of isoproterenol- and high $[K^+]_o$- stimulated taurine release from astroglia. ibid. 349-356.

Waniewski, R. A., Martin, D. L., and Shain, W. Isoproterenol selectively releases endogenous and [^{14}C]-labelled taurine from a single cytosolic compartment in astroglial cells. Glia (in press, 1991).

Book:

"Taurine: Functional Neurochemistry, Physiology and Cardiology", (H. Pasantes-Morales, D. L. Martin, W. Shain, R. Martin del Rio, eds.) Wiley-Liss, New York, 1990.