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Quantitative Analysis of Changes in Cell Volume and Intracellular pH Induced by Ammonia and Ammonium in Neuroblastoma Cells Studied with Fluorescent Probes

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system employs custom-made quartz AODs (positioning time <10 μ s; IntraAction) and is coupled with a UV-reflecting dichroic mirror to an inverted microscope fitted with a UV-transmitting objective lens (Fluar 100 \times , N.A. 1.3; Zeiss). Concurrent acousto-optical modulation (AOM) of beam intensity is used to control the pulse energy on a point-by-point basis and thus allows for graded photolysis. A monolayer of cultured hippocampal neurons was used to test this system. Bath-applied caged glutamate (200 μ M, CMB- γ cage; Molecular Probes) was photolyzed with single laser pulses (\sim 5 μ J at preparation), and receptor currents were measured in whole-cell voltage clamp during blockage of transmitter release with cadmium (50 μ M). AMPA and NMDA components were differentiated pharmacologically (25 μ M APV and 10 μ M CNQX). No response to multiple photolysis pulses was detected in the presence of both antagonists. Receptor currents could be graded by modulating the pulse energy over a restricted range. Future extensions include combining this micro-photolysis system with Random-access Fluorescence Microscopy to form a powerful tool for simultaneous optical stimulation and recording. (Supported in part by National Science Foundation grant IBN-9723871 to P. Saggau.)

28. Characterization of Quinoline Sulfonamide Zn-sensitive Fluorescent Probes for Use in Live Tissues VLADISLAV SNITSAREV and ALAN R. KAY, *Department of Biological Sciences, University of Iowa, Iowa City, Iowa* (Sponsor: T. Hoshi)

Ionic Zn is found at high concentrations (>100 μ M) in vesicles in some cells, notably in the terminals of some glutamatergic terminals in the mammalian forebrain. Two water-soluble fluorescent probes, Zinquin and TFLZn, have recently been developed based on the Zn²⁺ sensitivity of the quinoline sulfonamide moiety that have proved useful in studying vesicular Zn. We have characterized the interactions of these probes with Zn²⁺ in membranous systems using unilamellar liposomes. We have focused this study on Zinquin, which was found to have a higher quantum yield than TFLZn. Zinquin-free acid was found to cross membranes passively, obviating the need for an acetoxymethyl (AM) ester derivative for loading. The stoichiometry of the complexes formed between Zn²⁺ and Zinquin and the location of these complexes in liposomal solutions was studied by a combination of fluorescence spectroscopy and fluorescence lifetime measurements. In aqueous solution, the 1:2 [M/L] complex was favored. In liposomes, both 1:1 and 1:2 complexes were formed, both partitioning into the membrane, with the 1:2 complex able to act as an ionophore. The ionophoretic activity was blocked by a transliposomal pH gradient of \sim 2 U similar to that found across synaptic vesicles.

In live tissue, Zn²⁺ probes may diffuse across membranes passively or be actively taken up by a transporter or endocytosis. To distinguish between these pathways, the rate of Zinquin uptake was measured as a function of temperature, as below 15°C, endocytosis is blocked and active transport would be more severely retarded than passive transport. The uptake of Zinquin was measured in rat neocortical slices held in a constantly circulating oxygenated saline at 26 and 8°C. The time course of loading at both temperatures appeared biexponential, with slower rates at lower temperature. The Q_{10} for both processes was \sim 1.5, which is consistent with diffusion accounting entirely for the movement of Zinquin. (Supported by NIH grant NS-35243).

29. Cellular Distribution, Protein Interactions, and Aggregate Formation of Huntingtin's Amino Terminus Using GFP Variants and Fluorescence Resonance Energy Transfer (FRET) Analysis SHAWN D. HANDRAN, MARCY E. MACDONALD, and ANNE B. YOUNG, *Department of Neurology and Molecular Neurogenetics Unit, Massachusetts General Hospital, Boston, Massachusetts* (Sponsor: C. Gonzalez)

Huntingtin is a large protein of unknown function and contains a polyglutamine segment near the NH₂ terminus. DNA instability and expansion of the CAG tract that encodes the polyglutamine stretch results in Huntington's Disease (HD). The cellular dynamics of huntingtin and its putative interacting proteins were investigated by generating fusion proteins using the cyan (ECFP) and yellow (EYFP) variants of green fluorescent protein (GFP). EYFP was fused in-frame with truncated huntingtin (aa 1–550, termed HDN) containing normal (Q32) or mutant (Q100) polyglutamine segments. ECFP was fused in-frame to fragments of three different huntingtin yeast partners (HYP; Faber et al. 1998. *Hum. Mol. Genet.* 7:1463–1474): HYP A-ECFP, HYP B-ECFP, and HYP L-ECFP. COS cells from confluent cultures were singly or doubly transfected by electroporation with HDN Q32 or Q100-EYFP and/or HYP A, B, or L-ECFP constructs. Cellular distribution of HDN was largely cytoplasmic, with little or no nuclear staining. Some transfected cells exhibited HDN aggregates, which were also observed in cells transfected with HDN containing nonpathogenic range of glutamine repeats (Q32). Expression of HYP A-ECFP (a fragment of FBP11, a WW domain-containing splicing protein) alone appeared to be toxic to cells, as abnormal morphological appearance was observed over a period of several days after transfection. Cellular localization was nuclear and cytoplasmic. HYP B-ECFP, a WW domain-containing protein fragment of unknown function, was localized almost exclusively to the nucleus, with very little or no detectable cytoplasmic signal. HYP L-ECFP, a fragment of human FIP-2 protein containing a leucine zipper domain, was diffusely localized throughout the cytoplasm and nucleus. Preliminary results coexpressing HYP B-ECFP and HDN Q32 or Q100-EYFP show that there is almost no colocalization, and to date, redistribution of either has not been robustly observed, although some instances of altered HYP B-ECFP distribution were seen. HYP L-ECFP and HDN-EYFP have extensive overlap in the cytoplasm, but not within the nucleus. Interestingly, there is recruitment of HYP L-ECFP into HDN aggregates in cells exhibiting aggregate formation. Preliminary observation of FRET between HDN-EYFP and HYP B-ECFP was negative, perhaps due to the lack of cellular colocalization. Testing of FRET in additional pairings is ongoing, as is generation of full length protein constructs. ECFP was a gift of Dr. R.Y. Tsien. (Supported by the Hereditary Disease Foundation and NIH grants AG-13617 and NS-32765.)

30. Quantitative Analysis of Changes in Cell Volume and Intracellular pH Induced by Ammonia and Ammonium in Neuroblastoma Cells Studied with Fluorescent Probes VANIA D. ROSAS,[†] G. BOYARSKY,^{||} S. MARQUEZ,^{‡§} and F.J. ALVAREZ-LEEFMANS,^{*§§} *Department of Pharmacology, Cinvestav, IPN, Apartado Postal 14740, México D.F., México*, [†]*Department of Neurobiology, IMP, Av. México-Xochimilco 101 México D.F., México*, [§]*Department of Physiology and Biophysics, Wright State University, Dayton, Ohio*, and ^{||}*University of Texas Medical Branch, Galveston, Texas*

Elevated levels of ammonia (NH_3) and ammonium (NH_4^+) lead to neuronal and glial swelling and may contribute to the brain edema seen in various hepatic diseases. The Pathophysiology of this edema is not well understood. Moreover, exposure of cells to $\text{NH}_3/\text{NH}_4^+$ is a commonly used experimental maneuver aimed at displacing intracellular pH (pH_i) in a controlled and predictable manner to study its regulation and yet the volume changes associated with this maneuver are not well understood. Here, we show a method whereby simultaneous changes in cell water volume (CWV) and pH_i can be studied quantitatively in single cells. CWV and pH_i were measured simultaneously in single neuroblastoma cells (N1E-115 and NG108) loaded with BCECF. pH_i was measured ratiometrically, and CWV was determined at the isobestic wavelength (440 nm). Independent CWV measurements were done using calcein, a fluorescent probe insensitive to intracellular ions (Alvarez-Leefmans et al. 1995. *Methods Neurosci.* 27:361). Experiments were done with solutions buffered with Hepes without added bicarbonate. Upon exposure to 20-mM NH_4Cl isosmotic solutions, the cells swelled by $12.7 \pm 1.7\%$ above their initial CWV in 1.5 ± 0.1 min ($n = 8$) and then displayed isosmotic regulatory volume decrease (RVD). Upon removal of $\text{NH}_3/\text{NH}_4^+$, the cells shrank and recovered their initial volumes with a variable time course. Simultaneously recorded changes in pH_i suggest that most of the swelling may be due to intracellular NH_4^+ accumulation. The latter would result from protonation of intracellular NH_3 and by direct entry of NH_4^+ via channels or carriers. The recovery of the shrinkage after $\text{NH}_3/\text{NH}_4^+$ removal could be blocked by amiloride (1 mM), suggesting the involvement of Na^+/H^+ exchange in isosmotic regulatory volume increase. (Supported by NINDS grant no. 29227 and a Guggenheim Fellowship to F.J. Alvarez-Leefmans.)

31. Designing Photoregulated Ion Channels and Enzymes ANDREW WOOLLEY, A. JAMES, D. BURNS, A. GOVINDARAJAN, D. LIU, L. LIEN, and J. ZHANG, *Department of Chemistry, University of Toronto, Toronto, Canada* (Sponsor: B.M. Salzberg)

Photoregulated artificial ion channels would enable direct manipulation of cellular excitability using light. Photoregulated enzymes could be tools for dissecting complex cellular chemistry. We have prepared a first-generation photomodulated ion channel that is a derivative of the peptide gramicidin. This derivative has a COOH-terminal extension that is designed to act as a photogate at the entrance (and exit) of the channel. We have prepared a series of ribonuclease S analogues with photoisomerizable residues at various unique positions in the enzyme structure. Different types of photoisomerizable amino acids based on an azobenzene core have been synthesized: phenyl-azophenylalanine (Pap); 4-carboxy-phenyl-azophenylalanine (C-Pap), and a constrained derivative, phenylazo-1,2,3,4-tetrahydro-3-isoquinolinocarboxylic acid (Patic). The effects of these moieties on enzyme activity can be rationalized in structural terms using data derived for NMR and molecular modeling of the modified enzymes. The design strategy, modeled structures and observed activities of these analogues will be discussed.

32. Use of Optical Probes to Study Presynaptic Regulation of Monoamine Quantal Size in CNS Neurons and Secretory Cells EMMANUEL N. POTHOS, VIVIANA

DAVILA, and DAVID SULZER, *Departments of Neurology and Psychiatry, Columbia University, and Department of Neuroscience, New York State Psychiatric Institute, New York*

Recently, amperometric recordings have provided the means to detect presynaptic quantal release of monoamine transmitters from axonal varicosities and cell bodies (Wightman et al. 1991. *PNAS.* 88:10754; Zhou and Misler. 1995. *Biophys. J.* 39A:398; Pothos et al. 1998. *J. Neurosci.* 18:5575; Jaffe et al. 1998. *J. Neurosci.* 18:3548). We adapted these approaches to record quantal dopamine release from small synaptic vesicles in axonal varicosities of CNS neurons (Pothos et al. 1998. *J. Neurosci.* 18:4106).

As this approach records only transmitter released directly under the electrode, the use of markers to identify active presynaptic sites may improve data collection. We used FM1-43 ($4 \mu\text{M}$) to label presynaptic sites in midbrain dopamine neuronal cultures before and after exposure to secretagogues (40 mM K^+ , $20 \text{ nM } \alpha$ -latrotoxin). Neurons are initially maintained in Ca^{2+} -free media for 10 min, showing little decrement in FM1-43 label. If subsequent application of secretagogue decreases FM1-43 label at a site, we find that it is often a good candidate for amperometric recording. We have also used nicotine as a more selective secretagogue, as nicotinic receptors are highly expressed at dopamine presynaptic terminals. This axonal terminal localization of nicotine receptors was confirmed by fluorescent α -bungarotoxin (Oregon Green), which labels dopaminergic terminals in the living preparation with fairly good specificity, as seen by subsequent fixation and immunostain for tyrosine hydroxylase.

In amperometric recordings from cultured adrenal chromaffin cells, we examined the effects of stimulation on quantal size. We find that depolarization increases uptake of the fluorescent weak base vital dye acridine orange (AO) in chromaffin granules. Interestingly, both vesicular acidification and quantal size were increased by the nicotinic agonist DMPP ($10 \mu\text{M}$). Both effects were blocked by the removal of extracellular Ca^{2+} , the proton pump inhibitor bafilomycin, and the Cl^- channel blocker NPPB. The combination of AO fluorescence and amperometry suggests that vesicular acidification, which may depend on increased Ca^{2+} entry, increases quantal size. This effect may be due to potentiation of a vesicular Cl^- current.

33. Calcium Release Channels are Localized to Neurosecretory Granules in Terminals of the Mouse Neurohypophysis B.M. SALZBERG, M. MUSCHOL, S.D. KRANER, and A.L. OBAID, *Departments of Neuroscience and Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania*

The neurosecretory terminals of the vertebrate neurohypophysis have long provided an important model system for investigation of the mechanisms that mediate the rapid release of neurotransmitters and neuropeptides. From the hypothalamus (supraoptic and paraventricular nuclei in mammals; preoptic nucleus in amphibia), magnocellular neurons project their axons as bundles of fibers through the median eminence and infundibular stalk, to arborize extensively and terminate in the neurohypophysis, where the neuropeptides vasopressin and oxytocin are released into the circulation by a calcium-dependent mechanism. In the rat, some 2×10^4 neurons give rise to $\sim 4 \times 10^7$ neurosecretory terminals and swellings, whose membranes account for nearly 99% of the excitable membrane in the neurohypophysis (pars nervosa). The dense packing of terminals prob-