EFFECT OF NITRIC OXIDE REAGENTS ON INTRACELLULAR CALCIUM SIGNALING IN RBL-2H3 MUCOSAL MAST CELLS

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Abstract

Evidence has shown that intracellular calcium must be elevated in order for secretion of the granular contents within mast cells to occur. Exogenous nitric oxide (NO) has the effect of dampening the secretory responses of mast cells while the inhibition of nitric oxide synthase (NOS) amplifies the secretion. As elevation of intracellular calcium concentration is essential for secretion to occur, it is likely that NO inhibits secretion by activating calcium pumps leading to the removal of calcium from the cytosol. This research examines the role of nitric oxide in the regulation of antigen-activated RBL-2H3 mucosal mast cells and looks to determine which parts of the signal transduction pathway are involved in the activation and regulation of NO biosynthesis. Knowing that the NO-producing enzyme, nitric oxide synthase (NOS), is regulated by intracellular calcium, we will investigate whether a change in nitric oxide production affects intracellular calcium signals.

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List of abbreviations

A	
α	alpha
Ab	antibody
Ag	antigen
B	
β	beta
BH_4	tetrahydrobiopterin
BSA	bovine serum albumin
C	
Ca^{2+}	calcium ion
$[Ca^{2+}]_i$	intracellular calcium concentration
$CaCl_2$	calcium chloride
CaM	calmodulin
CO_2	carbon dioxide
D	
DAG	diacylglycerol
E	
EDRF	endothelium-derived relaxation factor
EDTA	ethylenediaminetetraacetic acid
Em λ	emission wavelength
ER	endoplasmic reticulum
Ex λ	excitation wavelength
F	
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
FMN	flavin mononucleotide
G	
cGMP	guanosine cyclic monophosphate
GTP	guanosine triphosphate

H

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

I

IBMX	3-isobutyl-1-methyxanthine
IgE	immunoglobulin E
IP_3	inositol-1,4,5-trisphosphate
IP_4	inositol-1,3,4,5-tetrakisphophate
IS	intracellular stores

J

K

KCl	potassium chloride

L

L-NA	L-nitroarginine

M

Mg^{2+}	magnesium ion
$MgCl_2$	magnesium chloride
ml	milliliter
mM	millimolar
Mn^{2+}	manganese ion

N

Nac	N-acetyl-L- (+) cysteine
NaCl	sodium chloride
Na_2CO_3	sodium bicarbonate
NADH	nicotinamide adenine dinucleotide (reduced form)
NaOH	sodium hydroxide
NBCS	newborn calf serum
nm	nanometer
NO	nitric oxide
NOS	nitric oxide synthase

O

O_2	oxygen

P

PIP_2	phosphotidylinositol bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate

PMT	photomultiplier tube

Q

R

RBL-2H3	rat basophilic leukemia cells
rpm	revolutions per minute

S

SMOCs	second- messenger-operated channels
SNP	sodium nitroprusside
SOCCs	store-operated calcium channels
SR	sarcoplasmic reticulum

T

Tg	thapsigargin

U

µl	microliter
µM	micromolar

V

W

X

Y

Z

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Chapter One

Introduction

The discovery of the mast cell occurred in 1877, when Paul Ehrlich, in testing some new basic anilin dyes for histological techniques, discovered cells in the connective tissue that contained large granules with a great avidity for these dyes and a tendency to alter the shade of blue ("metachromasia").[13] Because the cells are more numerous in connective tissue that have been well nourished, Ehrlich called them 'Mastzellen' or well fed cells. Ehrlich was able to describe the morphology of the mast cell, its distribution, its staining properties and the water solubility of its granules.[13] It was not until sixty years later, however, that the function of the mast cell was established. Jorpes, Holmgen and Wilander discovered that heparin, which also stains metachromatically with toluidine blue, is found in these cells.[13] Their findings of the close relationship between mast cells and the heparin content of tissues provided the conditions necessary for progress in the biochemistry of the mast cell.

Mast cells are strategically placed to detect ingested or inhaled antigens. They can be found scattered throughout the connective tissue, but are found primarily beneath the skin, just outside the gastrointestinal and respiratory tract, nasal mucous membranes and close to blood vessels and nerves. Tissues that are often exposed to trauma are especially rich in mast cells. In addition to an eccentrically located nucleus, they have 50–200 densely packed granules that contain relatively large amounts of chemical substances that are involved in the allergic response. The cells express large numbers of high affinity Fc receptors on their surface for the antibody (Ab) immunoglobulin E

(IgE). As a result, the mast cell is coated with IgE molecules that have been absorbed from circulation and serve as receptors for IgE-specific antigens (Ag) or allergens. *In vivo*, mast cells bind IgE molecules with a variety of antigenic specificities, making it likely that the cells are capable of responding independently to any of the antigens. The IgE receptor-activated increase in plasma membrane permeability to calcium is extremely long-lived, but the IgE receptors must remain aggregated for the pathway to be maintained.[4]

Activation of the mast cell occurs when a multivalent antigen cross-links two or more IgE molecules occupying Fc$\in$RI cell receptors found on the surface of the cell.[5] The resulting attachment of the Ag to the Ab causes the mast cell to become activated. This in turn initiates the cascade of events leading to an allergic reaction. Activation is characterized by granule swelling and solubilization of the crystalline structures within the granules, eventually leading to degranulation and the release of exceedingly large amounts of granular contents into the surrounding tissue. Some of the many different substances that are released immediately or secreted shortly thereafter include heparin, serotonin (an eosinophil and neutrophil chemotatic substance), bradykinin and slow reacting substance of anaphylaxis. They are responsible for causing local vascular and tissue reactions that initiate allergic reaction. These include such events as an increase in local blood flow due to arteriole dilation, attraction of the eosinophils and neutrophils to the reactive site, damage to the local tissue by the protease and contraction of local smooth muscle. Increased porosity of the capillaries also occurs, leading to leakage and an increase in permeability to plasma and fluid, thus inducing edema. Upon stimulation, cells also release superoxide dismutase and numerous acid hydrolases such as β-

hexoseaminadase, β-glucuronidase and arylsulfatase that act to degrade the extracellular matrix. Therefore, any number of different types of abnormal tissue responses can occur, depending upon the type of tissue in which the allergen-IgE reaction takes place.

Recent reports have shown that both the elevation of intracellular calcium, $[Ca^{2+}]_i$, and activation of protein kinase C are essential for secretion from the mast cell to occur in response to an antigen.[1,2] Receptor activation on the cell surface is coupled through a guanine nucleotide regulatory protein (G-protein) to the membrane bound enzyme, phospholipase C. (Figure 1) The activation of this enzyme catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 diffuses to the endoplasmic reticulum (ER) surface where it binds to an IP_3 specific receptor. The binding of IP_3 induces the opening of channels allowing Ca^{2+} ions to exit from the ER into the cytosol. Diacylglycerol, the other product of the PIP_2 hydrolysis, activates protein kinase C (PKC). The initial rise in cystolic calcium induced by IP_3 is thought to alter PKC so that it translocates from the cytosol to the cytoplasmic face of the plasma membrane. Once there, it is activated by the combination of calcium, DAG and a negatively charged membrane phospholipid, phosphatidylserine. PKC is found to be involved in the secretion process and may contribute to the replenishment of the intracellular calcium stores after the secretory response. PKC can also be activated by the phorbol ester, phorbol 12-myristate 13-acetate (PMA), but evidence has been shown that the activation of PKC alone does not induce secretion in RBL cells.[6] What is seen is a synergy between intracellular calcium increase and PKC activation.[6]

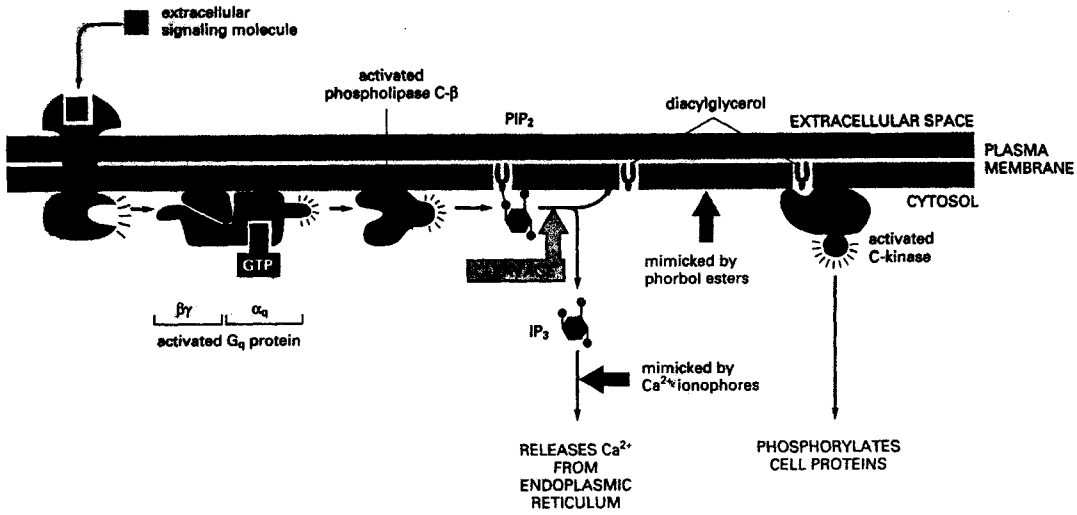


Figure 1: The two branches of the inositol phospholipid pathway[15]

Calcium enters the cytosol by depletion of intracellular calcium stores, such as the endoplasmic reticulum or by the influx of extracellular calcium. While the release of calcium from intracellular stores in mast cells may contribute to the increase in the $[Ca^{2+}]_i$ seen in response to Ag, it is generally acknowledged that the major source of calcium is extracellular calcium.[4] The total calcium content of resting RBL cells is in the region of 0.5 nmol/10^6 cells and this increases 5-6 fold on stimulation.[4]

The release of calcium from intracellular stores occurs via at least two families of channels that are similar to the ryanodine receptors located in the sarcoplasmic reticulum (SR) of muscle cells. The capacitative model of Ca^{2+} entry has been formulated to describe the linkage between calcium release from the stores and the activation of calcium entry.[7] The depletion of calcium stores can activate calcium influx across the plasma membrane. The channels responsible for the capacitative calcium influx when

the calcium stores are depleted are the store-operated Ca^{2+} channels (SOCCs). A second influx pathway is activated following membrane receptor activation, presumably by the generation of second messengers. These channels are known as the second-messenger-operated channels (SMOCs). Entry remains activated as long as the cells are stimulated and the stores remain depleted of calcium. Upon withdrawal of an agonist, IP_3 is rapidly hydrolyzed and inactivated by specific phosphatases, resulting in channel closure. Rapid entry from the outside continues until the calcium content of the IS reaches a level that inactivates calcium entry. Not all of the IP_3 is dephosphorylated however, some is phosphorylated further to form inositol-1,3,4,5-tetrakisphosphate (IP_4). IP_4 may be responsible for mediating slower and more prolonged responses in the cell to promote the refilling of the intracellular calcium stores from the extracellular fluid.[11] The reloading of IS occurs at resting $[Ca^{2+}]_i$ and when the reloading is complete, calcium entry is inactivated. This process is known as "capacitative calcium entry" and allows the release and entry of calcium to be controlled by a single messenger, IP_3.[7]

It is hypothesized that once the calcium enters the cell or is released from stores, activation of a pathway occurs to help maintain the intracellular calcium concentration. (Figure 2) First to be activated is the calcium-dependent enzyme, nitric oxide synthase (NOS), which is responsible for the oxidation of L-arginine to form L-citrulline and nitric oxide (NO). The physiological target of NO in smooth muscle is soluble guanylyl cyclase, where it binds to a heme iron. This enzyme is involved in catalyzing the reaction of guanine triphosphate (GTP) to yield the intracellular second messenger 3`,5`- cyclic guanine monophosphate (cGMP), which is responsible for activating the calcium ATPase pumps. Soluble guanylyl cyclase requires the presence of a physiological cofactor, either

Mg^{2+} or Mn^{2+}, plays a significant role in many physiological functions and can be found in vascular and brain tissues, platelets and liver cells.

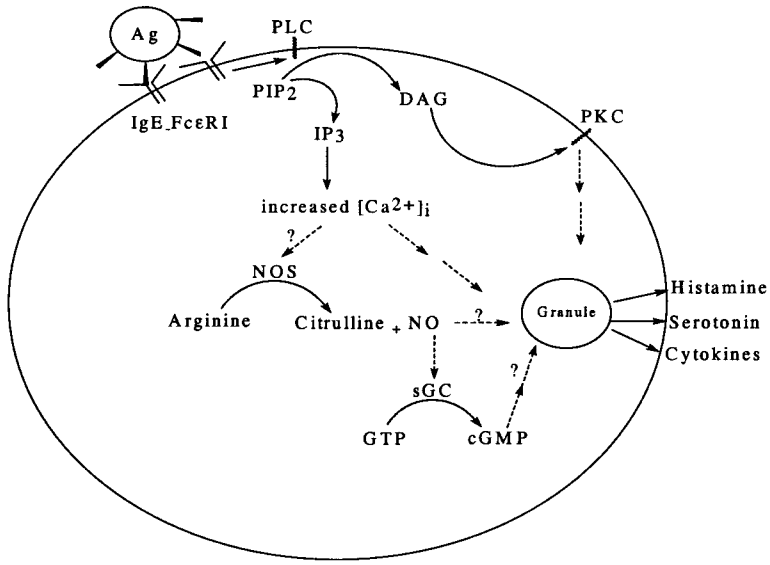


Figure 2:NO-Ca^{2+} Pathway

Nitric Oxide

The physiological and pathophysiological roles attributed to the actions of NO have grown tremendously since its identification as the endothelium-derived relaxation factor (EDRF) in 1987.[13] The small, short-lived, reactive and toxic free radical, NO, was once considered an atmospheric pollutant and bacterial metabolite. However, NO is now known to be involved in regulating blood vessel dilation, heart and skeletal muscle contractions, penile erectile function, neurotransmission and non-specific immunity. NO participates in the complex mechanism of tissue injury by perpetuating the inflammatory process. The diverse function of NO makes it a molecule of extreme pharmacological interest and physiological importance.

Normally, molecules acting as intercellular or intracellular messengers interact with specific receptor proteins on target cells to induce a necessary response. With NO being a radical and having lipophilic properties, it does not have a NO receptor and therefore does not follow the standard signaling process. What does occur is that it diffuses randomly away from where it is synthesized to interact with various intracellular molecules. The best characterized target site for NO is iron that is bound within certain proteins as heme or iron-sulfur complexes.

NO is enzymatically synthesized by NO synthase (NOS), which catalyzes the oxidation of L-arginine with O_2 to yield a 1:1 ration of NO and L-citrulline. (Figure 2) NO synthases are heme-containing proteins with a cytochrome P450-like active site. Three isomers of NOS have been identified consisting of two isoforms, constitutive and inducible. They differ in their dependence upon calcium and calmodulin. All three isoforms catalyze the same reaction and require the same NADPH and BH_4 cofactors, and contain FAD and FMN.

Each NOS subunit consists of about 125 to 160 kD and contains one FAD, one FMN, one BH_4 and one Fe (III)-heme. NOS is an active protein in its dimeric form, where its monomeric form lacks heme and BH_4, but retains its ability to bind CaM, FAD and FMN. The dimerization of the subunits seems to be promoted by heme, BH_4 and arginine, which besides their possible chemical roles, also appear to have a role in maintaining the integrity of the active protein.

Spectrophotometry

Spectrophotometry can be characterized as any procedure that uses light to measure chemical concentrations. Light is a form of electromagnetic radiation that can be described as a stream of discrete particles or wave packets of energy called photons or quanta. The energy of the photon is proportional to the frequency of the radiation and is given by the expression $E=h\nu$, where h= Planck's concept and ν = frequency.

When a molecule absorbs light, there is a transfer of energy to the medium, which decreases the radiant power of the beam of light. Each molecule has a series of closely spaced energy levels and when the energy of the molecule is increased due to light absorption, an electron is promoted from its lowest energy level (ground state) to a higher energy level (excited state). The electronic change yields a band spectrum of absorption that comprises the whole series of transitions: electronic, vibrational, and rotational.

In order for the transition of an electron from a lower energy level to a higher energy level to occur, the energy of the photon has to be exactly the same as the energy difference between the two orbital energies. The transition of an electron between two orbitals is called an electronic transition and the absorption process is called electronic absorption. The vibrational transitions come about because molecules have a multitude of quantized energy levels (or vibrational states) associated with the bonds that hold the molecule together. Rotational transitions are associated with the rotational motion around a molecule's center of mass. Each of the transitions is quantized and the overall energy associated with an orbital of a molecule is given by the following equation:

$$E = E_{electronic} + E_{vibrational} + E_{rotational}.$$

After a short period of time, if the molecule does not decompose as a result of the increase in energy and if all the energy is not dissipated by subsequent collisions with other molecules, the electron returns to the lower energy level while emitting a photon in the process. The time that a molecule stays in an excited state is characteristic of the atom or molecule and usually lasts for only 10^{-8} to 10^{-7} seconds. The transition from the excited state to the ground state involves the emission of energy. This short-lived emission is called fluorescence and is always observed at a lower energy than that of the absorbed excitation energy. A longer-lived emission of energy is known as phosphofluorescence, lasting 10^{-4} to 10^{2} seconds. The rationalization for fluorescence and phosphofluorescence being observed at a lower energy can be explained in Figure 3. A molecule absorbing radiation is in its ground state and possesses a certain geometry and solvation. When the radiation is first absorbed, the excited molecule still possesses its S_o geometry and solvation. Shortly after the excitation, the geometry and solvation revert to their most favorable values for the S_1 state. This lowers the energy of the excited molecule and when the S_1 molecule fluoresces, it returns to the S_o state but retains the S_1 geometry and solvation. This is an unstable configuration and must have a higher energy than that of the S_0 molecule with the S_0 geometry and solvation. The overall effect is that the emission energy is less than the excitation energy.

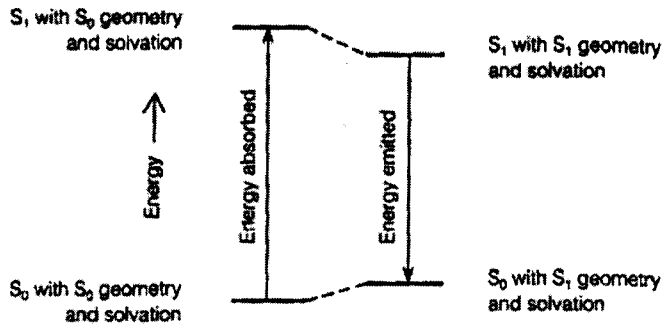


Figure 3: Diagram explanation of why absorbed energy is lower than emitted energy[18]

In order to understand why some molecules fluoresce and others phosphofluoresce, we have to under the properties of the electron. Electrons can spin about their own axis, having a spin, S, equal to (+/-) ½. A normal polyatomic molecule in its ground state usually has an even number of electrons with paired spins. The number of electrons with S= + ½ is equal to those with S= -½, resulting in a net spin equaling zero. The orbital angular momentum of a given state of an atom or molecule is termed its multiplicity. It is related to the electron spins where the multiplicity = 2S + 1. In molecules having all electron spins paired, S = +½ –½ =0, the multiplicity is 1. These molecules are said to have a singlet electronic state. Molecules can have an internal energy transition, which reverses the spin of a single electron in a polyatomic molecule. The molecule now finds itself with two unpaired electrons, S= ½ + ½ =1, each occupying a different orbital. The multiplicity term is 3, giving the molecule a triplet electronic level.

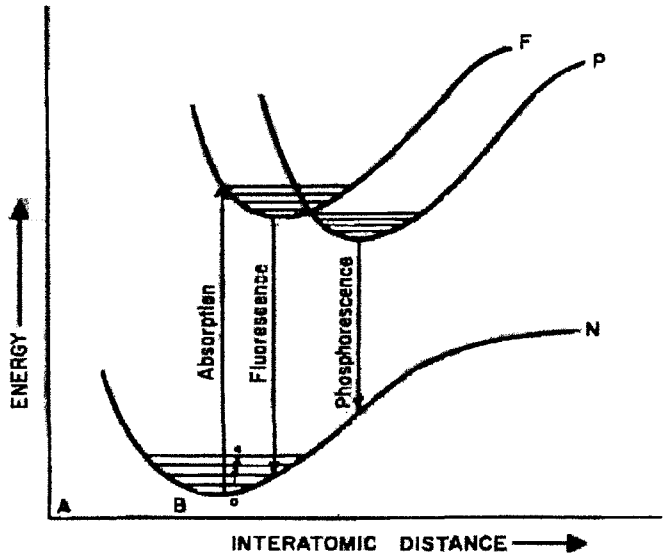


Figure 4: Potential energy of a diatomic molecule at various energy levels (according to Jablonski)[18]

According to Jablonski, Lewis and Kasha, most absorbing molecules have two excited states, F (singlet) and P (triplet), that are related to each other as shown in Figure 4. The transition from the ground state, N (singlet) to the excited state F (singlet), constitutes a normal absorption, where the reverse is known as fluorescence. A molecule in state F has a mean lifetime of the order of 10^{-8} seconds. Transitions from the excited state P (triplet) is called phosphofluorescence. The probability of transitions occurring directly from N to P is very small since N to P and P to N is a prohibited transition. Internal conversion (electron spin reversal) from F to P can possibly occur with some probability since the energy of the lowest vibrational level of P is lower than that of F. Molecules in the excited state, P, can return to the ground state, N, through F only by acquiring energy form the environment. Under these conditions the emitted light is the same as that produce by normal fluorescence, but the lifetime of the excited state is

longer than 10^{-8} seconds. Phosphofluorescence is therefore emission produced by the transition from a triplet state (unpaired electrons) to a singlet state (paired electrons)

Once the radiation used for exciting a molecule is removed, the fluorescence intensity of a solution of fluorescent molecules decays at a rate characteristic of a first order reaction. The relationship between the change in fluorescence with time and the original fluorescence can be expressed in the same exponential manner as radioactive decay: $I=I_0e^{-t/\tau}$ where I = fluorescence intensity at time t

I_o = maximum fluorescence intensity during excitation

T = time after removing the source of excitation

τ = average lifetime of the excited state

It would appear that all molecules that absorb light energy should fluoresce. But under ordinary conditions, even with sensitive and appropriate detecting devices, the fluorescence efficacy of most absorbing molecules is very low. Fluorescence is not as widespread as light absorption because of competition in the quenching processes tending to decrease the quantum yield of fluorescence. The quantum yield is the fraction of the absorbed energy that can be re-emitted as fluorescence. It is designated as, Φ, where it is equal to the number of quanta emitted divided by the number of quanta absorbed.

The type of interference that is responsible for most instances of non-fluorescence results from competitive electronic emission of radiation. The general term for the most common type of quenching is internal conversion. There are many types of internal conversion, one being the non-radiative transition (electron spin reversal) from the excited, singlet electronic level to a triplet level with somewhat lower energy.

The fluorescence phenomena, which has been around for a long time, are those which were excited by sunlight and occur in the visible region requiring no instrumentation. Modern applications of fluorescence detection began with the development of ultraviolet lamps. These have included various arcs and other devices leading up to modern high-pressure gaseous discharge lamps. All of the instruments designed to utilize fluorescence as a tool for quantitative assays are based on the same general principle and present similar problems. Figure 5 shows the minimum requirements for a spectrophotometric instrument.

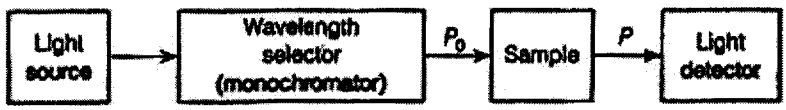


Figure 5: Schematic drawing of a monochromator[18]

The nature of the light source and its stability characteristics are important points to consider in an instrument. Light from a continuous source passes through a monochromator, which is used for isolating a narrow band of wavelengths from the incident beam. They are spectroscopic devices utilizing slits, lenses, or mirrors, and either prisms or grating for dispersing the radiation. The cells, or cuvettes used as containers for the solutions to be assayed, are important considerations since they can limit either the exciting or emitted light. They need to have windows fabricated from a

material that is transparent in the spectral region of interest. The best cells have windows that are normal to the direction of the beam to minimize reflection loss. Quartz is required for the ultraviolet region and about 3000 nm in the infrared. Silicate glass can be used for the region between 375 and 2000 nm and plastic can be used for the visible region.

The fluorescent light emitted by the sample comes to the detector along with scattered light and light emitted by the fluorescence of the container, the solvent and dissolved impurities. Removal of these interferences is carried out with suitable filter arrangements or by monochromators or combination of both. For a long time, the eye and the camera have been used as a means of detection, but modern instruments make use of photocells and photomultiplier tubes (PMT). The latter being the most sensitive device available for measuring light emission.

PMT is a vacuum tube in which there are many photosensitive electrodes arranged in series. Upon exposure to radiation, the PMT emits electrons that are accelerated towards a dynode maintained at a potential 90 V more positive than the cathode. Each accelerated photoelectron that strikes the dynode surface produces several additional electrons. These electrons are then accelerated to a second dynode, which is 90 V more positive than the first dynode. Electron amplification occurs again at the second dynode, and by the time this process has been repeated at each of the remaining dynodes, 10^6 to 10^7 electrons have been produced for each photon. This cascade of electrons is collected at the anode. The current pulses produced when photons strike the cathode are counted, and the accumulated count is a measure of the intensity of the

electromagnetic radiation impinging on the PMT. The PMT is consequently the amplifier as well as the detector.

Chapter Two

Literature Review

Xu, Star, *et al.* performed a multitude of experiments in order to show the importance of NOS and cGMP in their proposed model of "capacitative" calcium entry.[2] Their results, along with the findings of Pandol, *et al.* helped support their mechanism for the regulation of agonist-stimulated calcium entry.[8]

To test the possible involvement of the NO metabolic pathway in activation of calcium entry, rat pancreatic acini were treated with a specific inhibitor of NOS, an arginine analog known as L-nitroarginine (L-NA). Treatment of the cells with L-NA had no effect on resting $[Ca^{2+}]_i$ or the peak increase in $[Ca^{2+}]_i$ when the cells were excited by carbachol stimulation.[2] This suggested that L-NA had no effect on calcium release from IS. But what did occur was a fast diminishing of $[Ca^{2+}]_i$ and inhibition of Mn^{2+} entry in carbachol-stimulated cells, indicating that the treatment with L-NA inhibited Ca^{2+} entry.[2]

Xu, *et al.* reasoned that since NOS stimulation produces NO and the result is activation of guanylyl cyclase (GC) with an increase in cGMP, then cGMP must be important in the activation of Ca^{2+} entry and that inhibition of GC should also inhibit Ca^{2+} entry. Their results confirmed this idea and those of Pandol, *et al.* showing that GC inhibition using LY83583 had no effect on resting $[Ca^{2+}]_i$ or the initial increase in $[Ca^{2+}]_i$ following carbachol stimulation.[2] What did occur was inhibition of calcium entry and a fast reduction in $[Ca^{2+}]_i$ and reduced rate of Fura –2 quench by Mn^{2+}. Nine experiments were performed where the cells were pretreated with LY83583 and results showed that it inhibited agonist-stimulated Mn^{2+} by $96 \pm 12\%$.[2]

Exposure of the L-NA pretreated cells to NO and cGMP by using NO_2^- and Bt_2cGMP, was effective in reversing the inhibition of calcium entry.[2] The use of NO_2^- was not effective in reversing the inhibition due to LY83583, but Bt_2cGMP was able to reverse the inhibition in both agonist and thapsigargin (Tg, Ca-ATPase)-treated cells.

The studies were extended and the results showed that LY83583 also inhibited Ca^{2+} and Mn^{2+} influx triggered by Tg-dependent IS depletion and inhibited a NO-stimulated GC.[2] Addition of NO using NO_2^- or SNP to the cells did not relieve the inhibition of LY83583 when the calcium entry was stimulated by agonist or Tg. This is because the activation of GC occurs after the NO releasing step. These results help indicate that cGMP generated as a result of calcium release from IS is involved in the regulation of Ca^{2+} entry.

By using the NO producing drug, sodium nitroprusside (SNP), a dual effect of cGMP was uncovered. SNP is normally used in millimolar range in order to obtain maximal increase in cellular cGMP. When high SNP concentrations were used on control and L-NA treated cells to test calcium entry, what was found was that high concentrations of cGMP inhibited, rather than stimulated calcium entry.[2] Incubating the cells with 30 μM SNP was as effective as treatment with L-NA and LY83583 in inhibiting calcium entry. It is likely that when $[Ca^{2+}]_i$ is kept at high levels by calcium entry, NOS activity increases cGMP to an inhibitory concentration. This high cGMP concentration will inhibit entry to reduce NOS activity and cGMP to activating levels. This mechanism can provide dynamic control of Ca^{2+} entry and guard against saturation of the cytosol with calcium. Inhibition of calcium entry and cGMP production by L-NA

in stimulated cells and the effect of SNP on entry enabled the determination of the relationship between cGMP and calcium entry in the stimulatory and inhibitory range.

The most compelling evidence to support the proposed model of Ca^{2+} entry of Xu, *et al.* is that the Ca^{2+} release from the IS is necessary and sufficient to activate NOS and cGMP production. It was shown that when the IS were kept fully loaded with Ca^{2+} and the medium $[Ca^{2+}]_i$ was increased up to 2.5 μM, NOS was only partially activated.[2] However, NOS was strongly activated when the IS was depleted while buffering $[Ca^{2+}]_i$ with EGTA.

The present studies provide evidence that calcium release from IS activates a Ca^{2+}-dependent enzyme, NOS, which is responsible for generating NO from arginine.[2] NOS activity is regulated by the calcium content in the IS, and is responsible for activating GC to generate cGMP which is responsible for modulating calcium entry.

Millard, Fewtrell, *et al.* studied the rise in intracellular free Ca^{2+} in RBL cells produced by aggregation of IgE receptors. Results show that when the RBL cells were stimulated with antigen, the average $[Ca^{2+}]_i$ increased sharply in all of the cells after lag times that varied in duration.[5] There were individual spikes, oscillations, in $[Ca^{2+}]_i$ shown which had a train appearance rather than a sinusoidal one and the rising and falling of each spike was approximately equal. Results using a calcium ionophore at levels that were nontoxic to the cells but would raise the $[Ca^{2+}]_i$ to a concentration that might be attained during antigen stimulation, showed that elevation of $[Ca^{2+}]_i$ using the ionophore did not induce oscillations. From their results they further concluded that not only are the antigen-stimulated $[Ca^{2+}]_i$ oscillations in RBL cells due to the influx of

calcium, but that the release of calcium from intracellular stores is a significant contributor.

It has been shown in a number of different cell types that fluctuations in the membrane potential are accompanied by oscillations in $[Ca^{2+}]_i$. The calcium influx in RBL cells is reduced when the cells are depolarized and depolarization occurs as a result of stimulation with an antigen. With this, Millard, *et al.* thought that it might be possible that the variations in the membrane potential could give rise to the oscillations in $[Ca^{2+}]_i$. What they found was that the oscillations in $[Ca^{2+}]_i$ still occurred in the absence of a membrane potential due to the RBL cells being depolarized using K^+, but were short-lived.[5] They suggest that since the influx is inhibited under these conditions that prolonged oscillations might not occur due to the IS stores becoming depleted and are unable to release calcium if they are not reloaded with extracellular calcium. The appearance of the $[Ca^{2+}]_i$ oscillations are not necessarily a result of transient depolarization of the plasma membrane. However, these results do not exclude the possibility that fluctuations in the membrane potential may enhance or even follow oscillations in $[Ca^{2+}]_i$ under certain conditions.

When calcium influx was inhibited using La^{3+}, even in the presence of extracellular calcium, transient oscillations in $[Ca^{2+}]_i$ occurred, but with no sustained elevation in $[Ca^{2+}]_i$.[5] This suggests that the release of calcium from intracellular stores is involved in generation of the $[Ca^{2+}]_i$ signals and that calcium influx is required for the sustained elevation and for continued oscillations in $[Ca^{2+}]_i$. They contribute the following explanation for their results. Oscillations in $[Ca^{2+}]_i$ are the primary result from the release of calcium from the stores, whereas the sustained increase in $[Ca^{2+}]_i$ is due to

the direct influx of calcium across the membrane and into the cytosol. The refilling of the stores is required to sustain the oscillations for more than 1-2 minutes, which is also dependent on calcium influx into the cell.

In an attempt to correlate the secretion of mast cells and calcium concentration, Kim, *et al.* showed that secretion in response to Ag never occurs until intracellular calcium is elevated.[1] In cells where the intracellular calcium concentration oscillates, secretion is seen around the peaks of the oscillations while being absent between oscillations.[1] Despite oscillations near resting levels reaching or exceeding levels normally seen in secreting cells, secretion is rarely seen. However, excellent secretion is seen during sustained, non-oscillatory increases in intracellular calcium. Secretory events in the mast cells do not appear to be specifically linked to either the rising or falling of the oscillations caused by the changing intracellular calcium concentrations. There is no obvious threshold concentration of calcium for the initiation of secretion and secretion is not detected during every increase in intracellular calcium concentration.

It is well known and widely accepted that IP_3 is the intracellular messenger that links cell surface receptor stimulation to the release of an intracellular Ca^{2+} pool.[8] But beyond this step, not much is known about calcium mobilization. Pandol, *et al.* using saponin-permeabilized rat parotid acinar cells, showed that intracellular calcium release due to IP_3 appears to be a "quantal" or incremental process.[8] This is clearly seen in their experiments with permeabilized cells where it has been shown that it is the fraction of the IP_3-sensitive calcium pool released, and not the rate of the total pool, that is dependent on the IP_3 concentration. Results from other experiments by Pandol showed that following the rapid release of calcium by IP_3, the calcium was sequestered. And after sequential

additions of submaximal concentrations of IP_3, occurred sequential calcium release. Poorly metabolized IP_3 analogues, (1,4,5) IPS_3 or (2,4,5) IP_3 and conditions where the metabolism of the authentic IP_3 was reduced were used. Results showed that calcium reuptake once again occurred, but sequestered calcium was not released by the subsequent additions of (1,4,5) IP_3.[8]

Studies have shown that the number of receptors that occupy antigen-specific IgE, does not have to be great in order to attain a maximal secretory response in rat basophilic leukemia (RBL) cells.[4] These results and others came from experiments done by Fewtrell, *et al.* showing that a maximal secretory response and calcium influx can be obtained when as few as 10% of the receptors are occupied with antigen-bound IgE. Calcium influx was significantly reduced when less than 10% of the IgE receptors were able to bind Ag, but only a slight increase in ^{45}Ca occurred when 30% or 100% of the receptors were available for Ag interaction. This showed that calcium influx correlates more closely with [3H]-serotonin secretion that than to the number of IgE receptors. This suggested that the extent of secretion was limited by the rate of calcium influx into the cells and that there is a substantial pool of "spare" receptors.[4]

Experimental

Materials & Methods

The RBL-2H3 cells were purchased from ATCC, mIgE-αDNP was donated by Dr. Juan Rivera (NIH) and Dr. Clare Fewtrell (Cornell University) donated the $DNP_{32}BGG$. Eagle's minimum essential medium and supplemental materials (fetal bovine serum (FBS), newborn calf serum (NBCS), anti-PPLO, penicillin, glutamine) and trypsin-EDTA were all purchased through Fisher Biotech. Components of Tyrode's saline solution were purchased from Fisher Scientific (NaCl, $MgCl_2$, HEPES), Mallinckrodt (KCl), Baker Chemical ($CaCl_2$) and Sigma (glucose, sulfinpyrazone, bovine serum albumin (BSA), gelatin). Reagents used for calcium concentration and β-hexoseaminidase experiments were obtained either through Sigma (L-NAME, IBMX, 8-BrcGMP, Imidazole, 4-methylumbelliferyl-N-acetyl-β-D-glucosamide, fura-2-acetoxymethylester) or Fisher Scientific (Triton-X-100, trypan blue, acetic acid, NaOH, glycine, Na_2CO_3, SNP, N-acetyl-L (+)-cysteine (Nac)).

RBL-2H3 cells were maintained in a monolayer culture in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 8% newborn calf serum, anti-PPLO, penicillin and glutamine. Cells were grown in 75-cm^2 tissue culture flasks and kept in incubation at 37 °C and in the presence of CO_2. Weekly cell passages were performed, seeding cells at different concentrations and adding supplemented medium for a total volume of 20.0 ml.

Cell passages and all pre-experimental treatments were performed under sterile conditions. Buffers and reagents used for both $[Ca^{2+}]_i$ and β-hexoseaminidase experiments were maintained at 37 °C unless otherwise noted. When cell counts were

obtained, both viable and non-viable cells were accounted for. An aliquot of a 1:1 ratio (100 µl: 100 µl) of cell suspension and trypan blue was applied to a hemacytometer, counting the cells located in the 4 outer squares. The following equation was used to calculate the total number of cells:

Of cells counted ÷ 200 x 10^6/ml x total volume of cell suspension = total # of cells

Volume per square is 0.1 µl: 0.1 x µl = 0.4 µl

0.4 µl/2 (dilution of cells) = 0.2 µl of original volume counted

$[Ca^{2+}]_i$ Assay

For the calcium concentration experiments, cells were sensitized using Eagle's essential medium containing 1.0 ug/ml of mIgE-αDNP and incubated overnight at 37 °C. The adherent cells were placed in suspension following a brief rinse and 10 minute incubation with trypsin-EDTA. Cells were dislodged and the reaction was quenched using sterile wash (Eagle's essential medium deficient in glutamine and FBS). Cell suspension was centrifuged at 1000 rpm, room temperature and for 10 minutes. The supernatant was discarded and cells were resuspended in an aliquot of buffered Tyrode's saline solution containing 135 mM NaCl, 5 mM KCl, 1.8 mM $MgCl_2$, 5.6 mM glucose, 10 mM Na-HEPES, 50 µM sulfinpyrazone and 0.1% BSA (pH 7.4). A cell count was obtained and the remaining cell suspension was centrifuged again at 1000 rpm, at room temperature and for 10 minutes. The supernatant was removed and cells were resuspended at a concentration of 1.0x10^6 cells/ml in the buffered Tyrode's saline solution. The cells were loaded with fura-2/AM at a concentration of .25 µM and incubated in a 37 °C water bath with shaker for one hour. Following incubation, a second cell count was obtained, cell suspension was centrifuged as above and cells were

resuspended to a concentration of $1.0x10^6$ cells/ml in Tyrode's saline solution containing 0.05% gelatin (pH 7.4) in place of 0. 1% BSA. Cells were now ready to be assayed.

Experiments were designed where the changes in fluorescence of fura-2/AM, due to $[Ca^{2+}]_i$, was monitored over a period of time using 3 ml aliquots of cell suspension and the Shidmadzu R5000 Spectrofluorophotometer equipped with a constant temperature cell holder and stirrer. Fura-2/AM was used as the calcium ion indicator, having an excitation wavelength of 334 nm and a detectable fluorescence at 510 nm. Nitric oxide generating reagents, NOS inhibitors and other pharmacological agents involved in either increasing or decreasing $[Ca^{2+}]_i$ were used in a variety of combinations on antigen-activated and unactivated mast cells sensitized with mIgE-αDNP.

The parameters on the spectrofluorophotometer for the $[Ca^{2+}]_i$ assays were set at the following:

Band Width (nm): Ex = 10.0 Em = 10.0	Sensitivity: High
Scan Mode: Time	Cell Position: 1
Fixed λ (nm): Ex = 334.0 Em = 510.0	File Res: Fine
Time Interval (sec): 0.5	Shutter Cont: Auto
Response (sec): Auto	

β-hexoseaminidase Assay

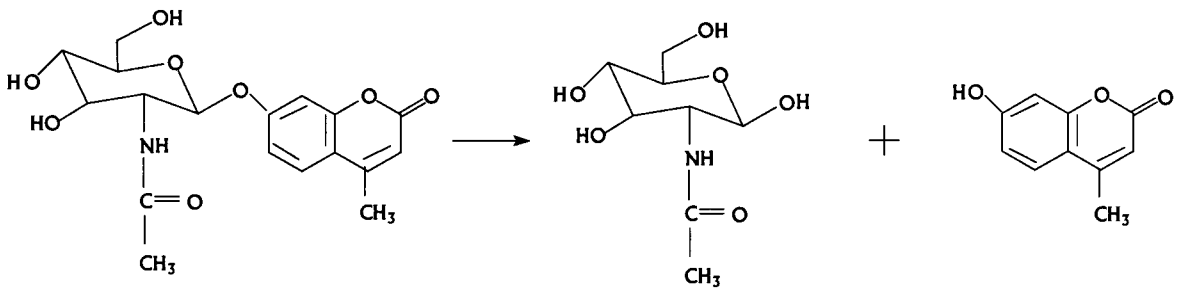
Cell preparation for the β-hexoseaminidase secretion assay included placing adherent cells in suspension using a brief trypsin-EDTA treatment followed by 10-minute incubation at 37 °C with trypsin-EDTA. Cells were dislodged, reaction was quenched and a cell count was obtained. Cell suspension was centrifuged at 1000 rpm, room

temperature for 10 minutes, and cells were resuspended at a concentration of $1.3x10^6$ cells/ml in supplemented Eagle's essential medium. Cells were sensitized with mIgE-αDNP at a concentration of 0.6 μg/ml and incubated overnight at 37 °C in sterile multi-welled plates. Following incubation, the welled-plates were placed in a 37 °C water bath and remained there throughout the assay. Each well was briefly washed with warm Tyrode's saline solution and cells were treated with a 30-minute incubation using varying concentrations of SNP made up in Tyrodes - (+/-) Nac. A duplicate set of wells was used throughout the experiment and addition of the solution took place at 30-second intervals. After incubation, Tyrodes-SNP (+/-) Nac was removed and secretion was activated by addition (30-second time intervals) and 30-minute incubation with antigen (1.0 μg/ml in Tyrode's solution- (+/-) Nac). Afterwards, the reaction was quenched, in 30-second time intervals using ice cold Tyrode's saline solution (-Mn^{2+}, Ca^{2+}). Four wells were left untreated, two for the 0% secretion and two for 100% secretion. The 50% wells were treated with addition of 10% triton, then quenched with ice cold Tyrode's. The secretory response was determined by the β-hexoseaminidase activity released from the cells. Aliquots were removed from each well and enzyme activity was monitored using 4-methylumbelliferyl-N-acetyl-β-D-glucosamide as the substrate. Substrate was prepared using a 1:100 dilution with acetate buffer. Following a 30-minute incubation, the reaction was quenched using glycine-carbonate buffer and 3 ml aliquots were then assayed fluorometrically on the same x-axis, using the Shidmadzu R5000 Spectrofluorophotometer for enzyme product. Aliquots were diluted with 1.0 ml of quench prior to fluorescence. Secretion was expressed as a percent of the total β-hexoseaminidase content of the cells before stimulation.

The parameters on the spectrofluorophotometer for the β-hexoseaminidase assays were set at the following:

Band Width (nm): Ex = 5.0 Em = 5.0	Sensitivity: High
Scan Mode: Time	Cell Position: 1
Fixed λ (nm): Ex = 360.0 Em = 450.0	File Res: Fine
Time Interval (sec): 1.0	Shutter Cont: Auto
Response (sec): Auto	

The concept of using the β-hexoseaminidase assay is based on the principle that the amount of β-hexoseaminidase released from the cell corresponds to the amount of NO produced, and thus $[Ca^{2+}]_i$. β-hexoseaminidase is responsible for catalyzing the reaction in Figure 6, and the amount of enzyme released is detected by the intensity of the fluorescent product (4-methylumbelliferone).



4-methylumbelliferyl-N-acetyl-β-D-glucosamide 4-methylumbelliferone

Figure 6: β-hexoseaminidase catalyzation reaction

Chapter 3

Results & Discussion

$[Ca^{2+}]_i$ Assays

The role of NO in Ca^{2+} regulation was determined without the other events that lead to secretion since PMA was not activated. Secretion of mast cells does not occur unless there is simultaneous treatment with the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA). The pharmacological reagents selected for the assays were used at varying concentrations, and several elicited contrary results.

In those assays where the cells were stimulated with antigen, $[Ca^{2+}]_i$ did increase with varying lag times. Figures 7 & 8 show the Ca^{2+} response of Ag-activated and unactivated mast cells treated with a NO-producing drug, SNP. When Ag-activated and unactivated mast cells were exposed to millimolar concentration SNP, an immediate decrease in the intracellular calcium concentration occurred. This coincides with results obtained by Xu, *et al.* that in order to obtain maximal increase in cellular cGMP, SNP is normally used in the millimolar range. Trials were done using micromolar concentration SNP, but no detectable decrease in $[Ca^{2+}]_i$ was observed (data not shown).

Figures 9 & 10 are fura-2 responses obtained when Ag-activated and unactivated mast cells were exposed to a NO-inhibiting reagent. Imidazole binds to the active site of NOS, blocking the function of the enzyme. These results show an increase in $[Ca^{2+}]_i$ for both Ag-activated and unactivated mast cells when treated with molar concentrations of Imidazole. Addition of SNP following exposure to Imidazole was successful in reducing the increase in $[Ca^{2+}]_i$ brought on by the inhibition of NO production. (Figures 11 & 12) Resting $[Ca^{2+}]_i$ was restored with the unactivated cells, but the $[Ca^{2+}]_i$ remained above

resting levels for the Ag-activated cells. Other trials were done using millimolar concentrations of Imidazole, giving contrary results to those obtained from use of a higher concentration (data not shown).

A substrate analog, L-NAME, responsible for inhibiting NO production via NOS was used on both Ag-activated and unactivated mast cells. Exposure to L-NAME brought about an immediate sharp increase in $[Ca^{2+}]_i$ with a relative rapid recovery. (Figures 13 & 14) The fura-2 response in Figure 14 shows repetitive spiking due to three sequential additions of L-NAME. These results parallel those found in literature, where Xu, *et al.* showed that calcium release from IS due to agonist or Tg activation increased $[Ca^{2+}]_i$, resulting in a 10-fold increase in cGMP which was inhibited by L-NA and L-NAME.[2]

Inhibition of phosphodiesterases in the cytosol was accomplished using IBMX. An expected decrease in $[Ca^{2+}]_i$ was seen when Ag-activated and unactivated mast cells were treated with a 1:10 dilution of millimolar IBMX. (Figures 15 & 16) When higher concentrations of IBMX were used, the opposite result occurred. Greater than millimolar and molar concentrations of IBMX increased $[Ca^{2+}]_i$, as seen in Figure 17. (Fura-2 responses from higher millimolar concentration IBMX are not shown.)

Figures 18 & 19 show responses obtained when Ag-activated and unactivated mast cells were treated with a cGMP analog, 8-BrcGMP. A decrease in $[Ca^{2+}]_i$ occurred for both, which agrees with the findings of Xu, *et al.* that inhibition of both GC and cGMP production inhibited calcium entry when measured directly or by Mn^{2+} influx into agonist-stimulated cells.[2]

Results from these assays helped to identify the relationship between NO and $[Ca^{2+}]_i$ in mast cells. The Ca^{2+}-NO pathway seemed to be activated even when an increase in $[Ca^{2+}]_i$ was due to the release by IS, and not by agonist stimulation. NO was found to be necessary to decrease $[Ca^{2+}]_i$ in stimulated mast cells and also in reducing the resting $[Ca^{2+}]_i$. When NO synthesis was hindered by either an NOS inhibitor or a non-functional arginine analog, the $[Ca^{2+}]_i$ increased due to inability of the regulation pathway to be furthered activated. Resting $[Ca^{2+}]_i$ levels were unable to be maintained due to the blocking of NO synthesis, which could mean that regulation of the calcium entry needs to be maintained even when cells are not stimulated. Without the production of NO, cGMP could not be increased to levels which enable it to modulate calcium entry. NO synthesis was restored to those cells using SNP, suggesting that the pathway could be activated by exogenous NO when endogenous NO is lacking. The peaks obtained from the use of L-NAME suggest a relatively rapid recovery of the pathway not seen in Imidazole-treated mast cells. A more precise reason for the spiking and fast recovery occurring with L-NAME could not be made at this time.

The conflicting results obtained when varying the concentration of the reagents may imply that something else may be taking place within the regulation pathway which were unable to be determined using the latter assays.

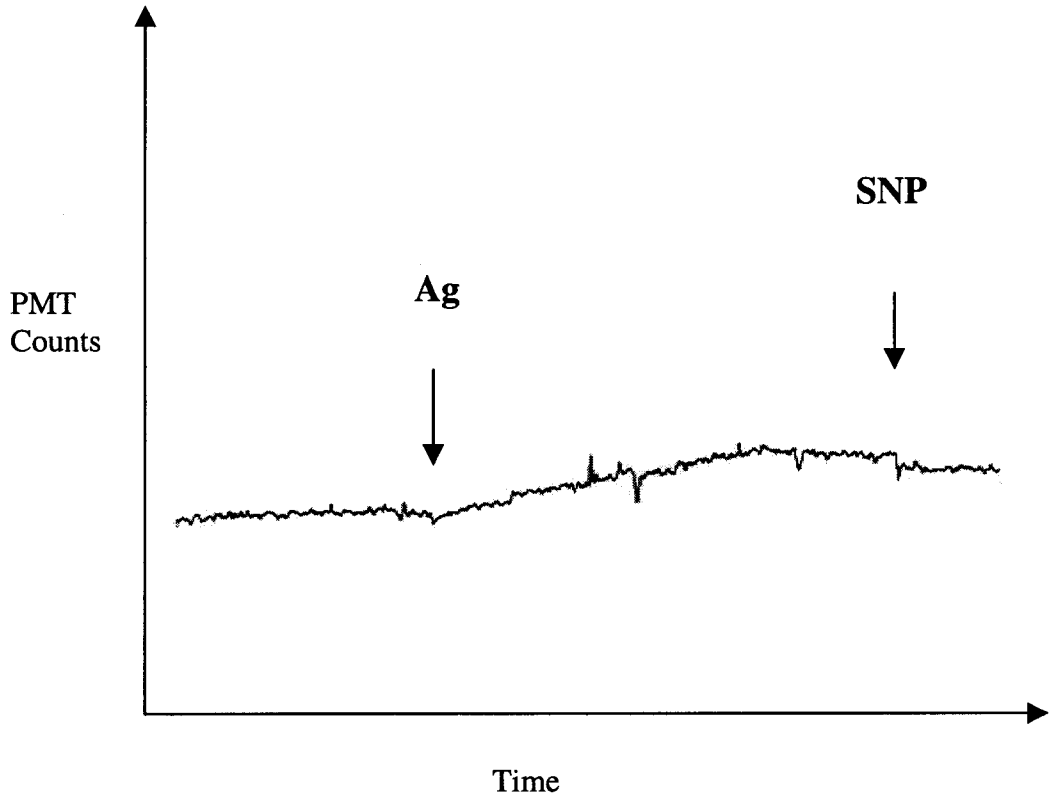


Figure 7: Spectrum of Ag-activated mast cells treated with 1 M SNP

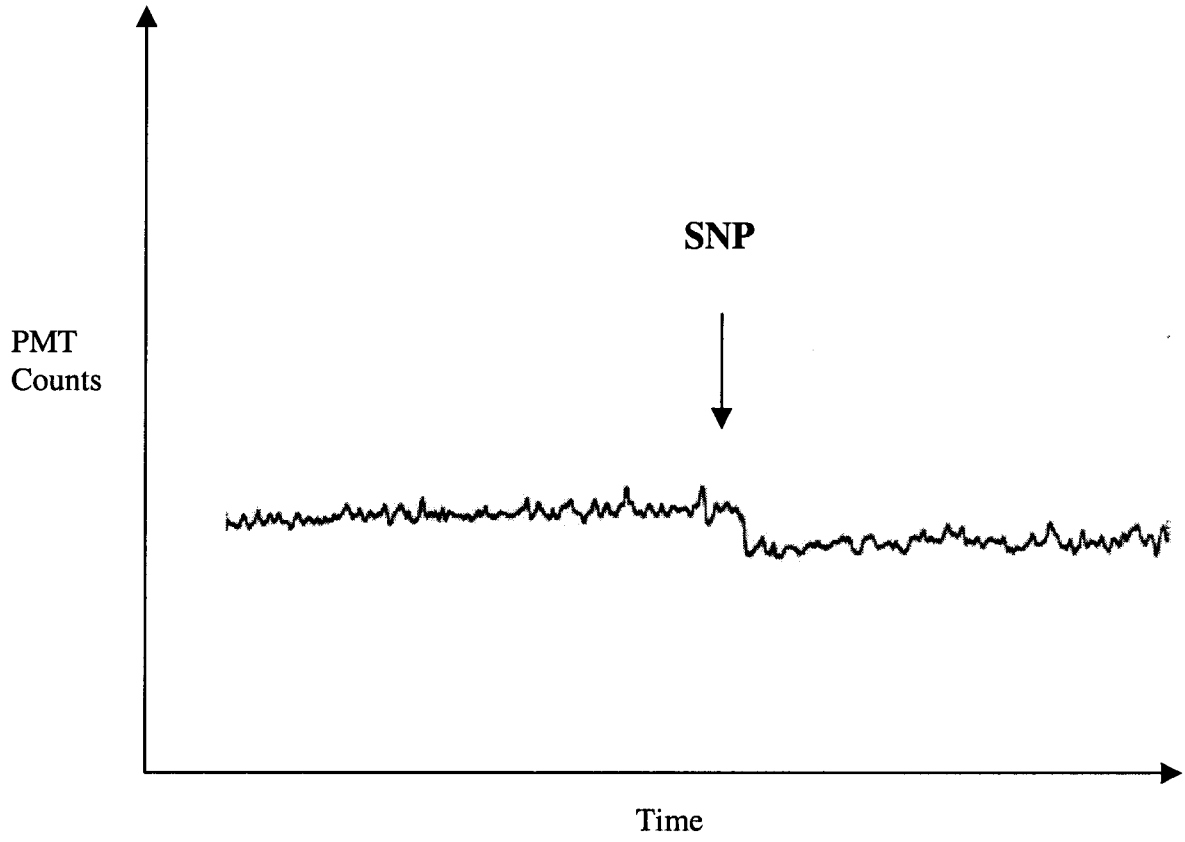


Figure 8: Spectrum of unactivated mast cells treated with 1 M SNP

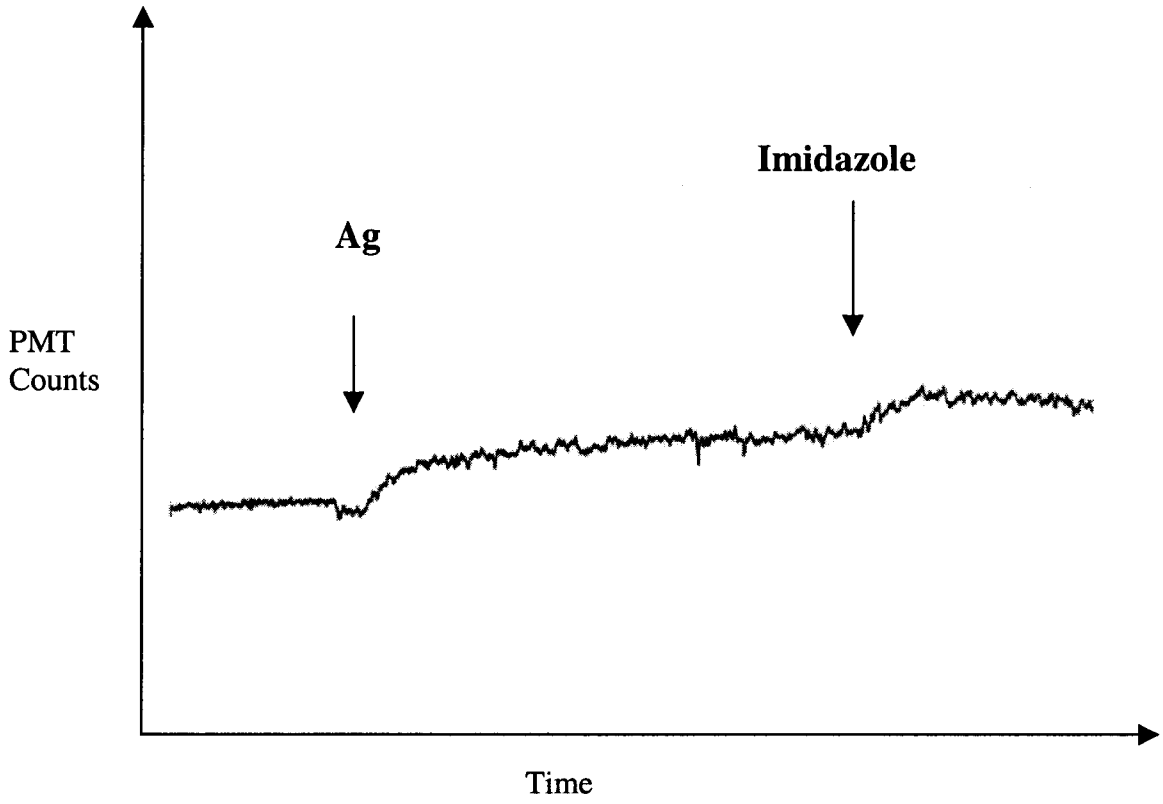


Figure 9: Spectrum of Ag-activated cells treated with 1 M Imidazole

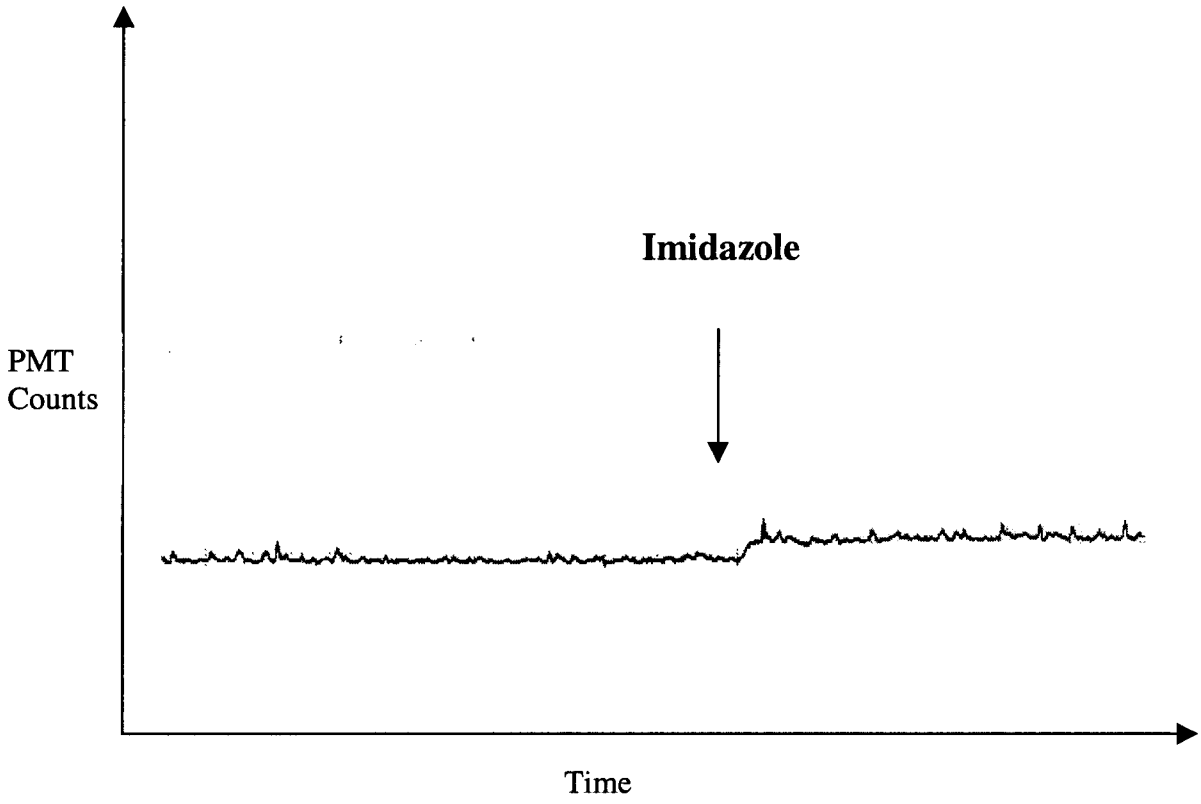


Figure 10: Spectrum of unactivated mast cells treated with 1 M Imidazole

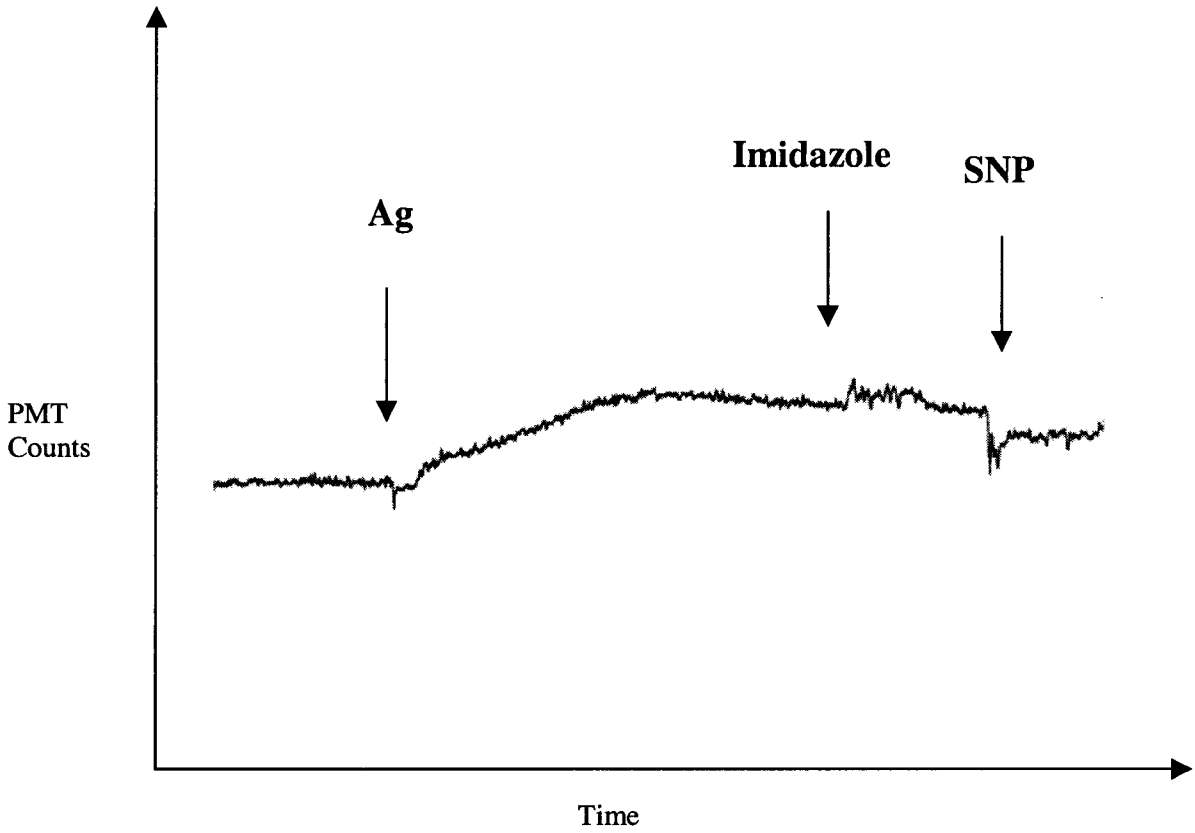


Figure 11: Spectrum of Ag-activated mast cells treated with 1 M Imidazole followed by treatment with 1 M SNP

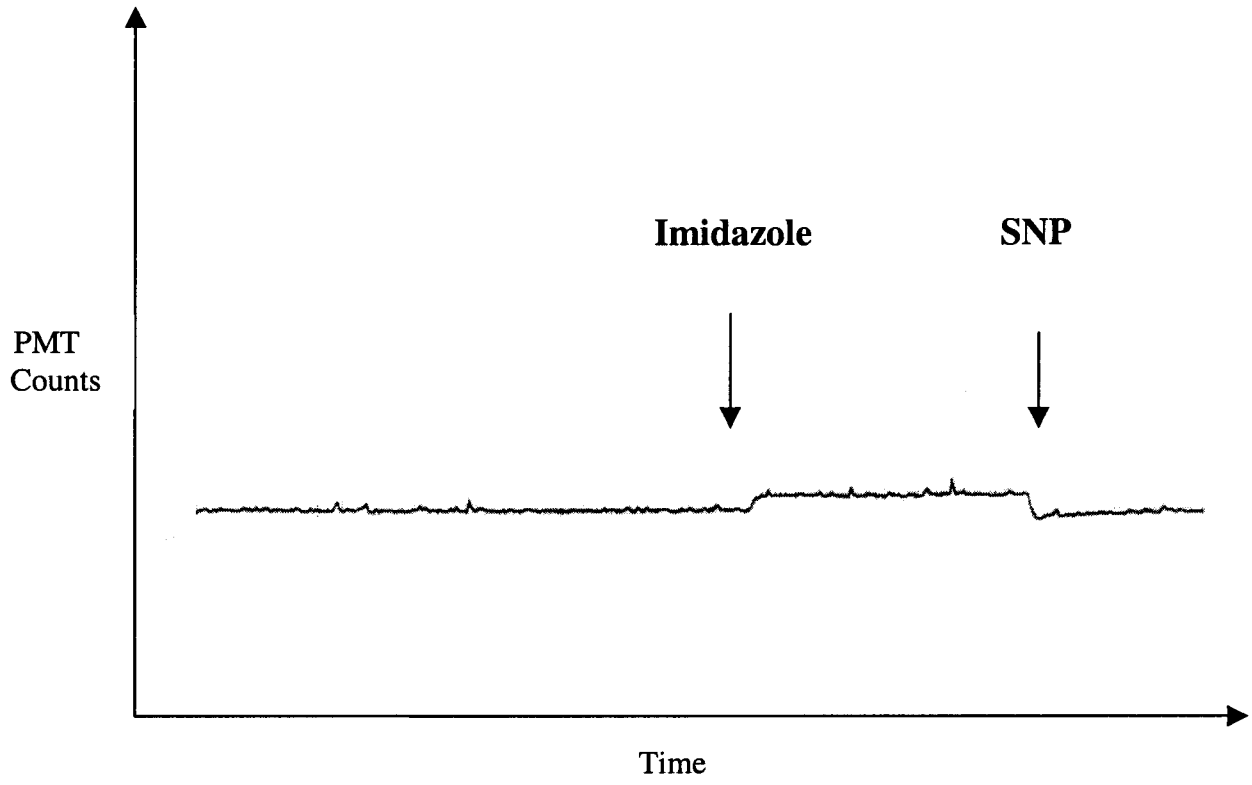


Figure 12: Spectrum of unactivated mast cells treated with 1 M Imidazole followed by treatment with 1 M SNP

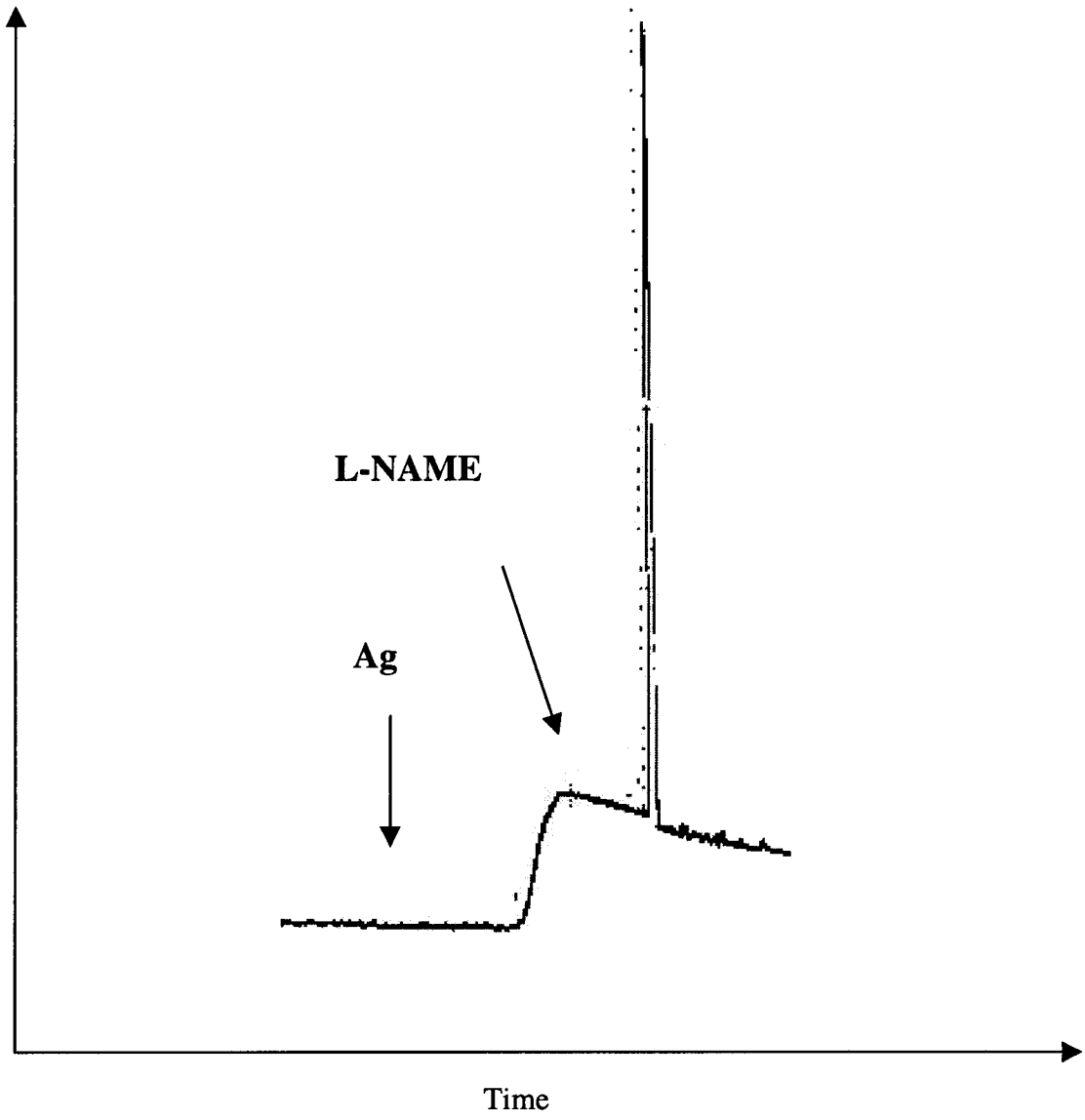


Figure 13: Spectrum of Ag-activated mast cells treated with 10 mM L-NAME

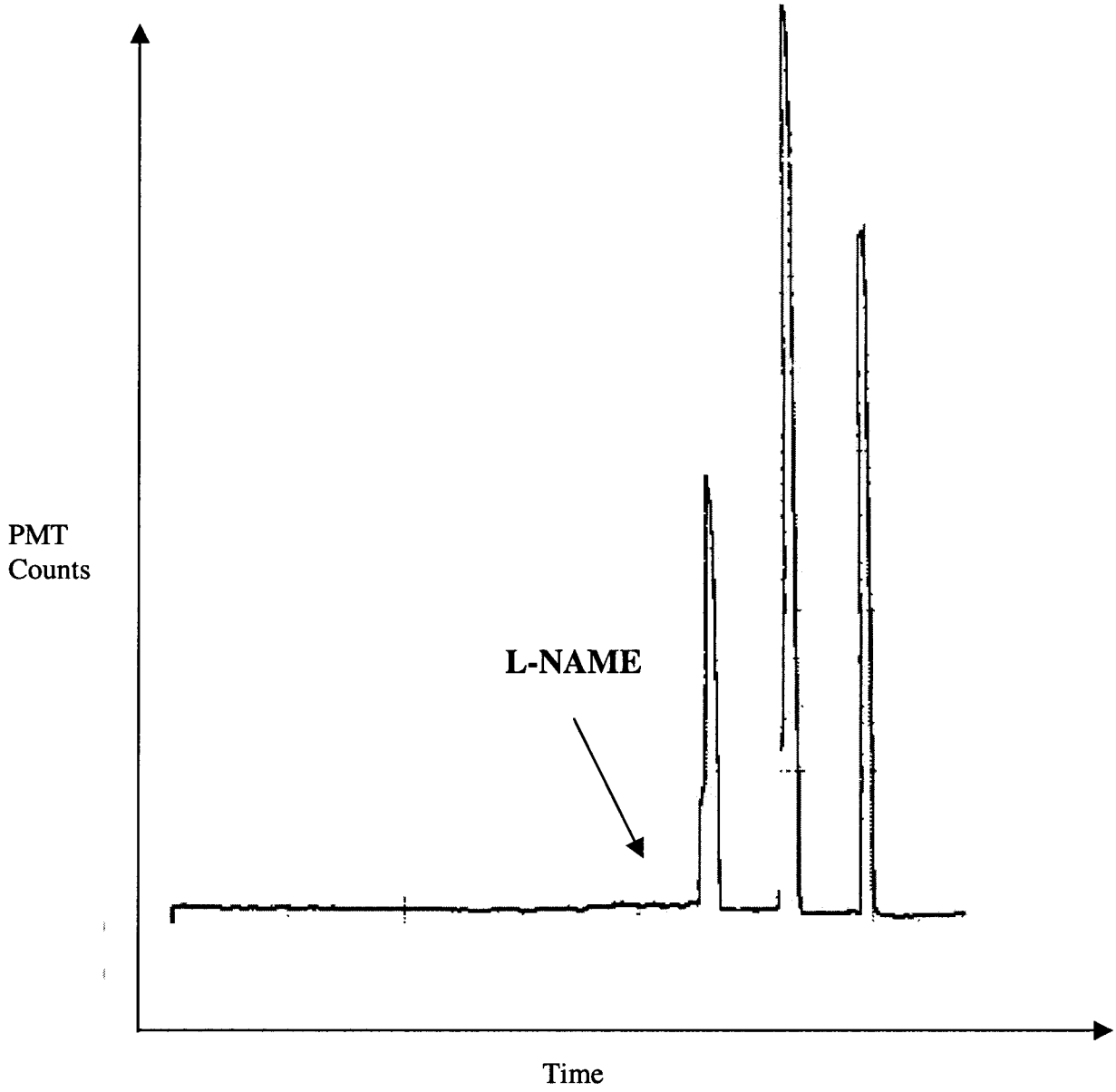


Figure 14: Spectrum of unactivated mast cells treated with three sequential additions of 10 mM L-NAME

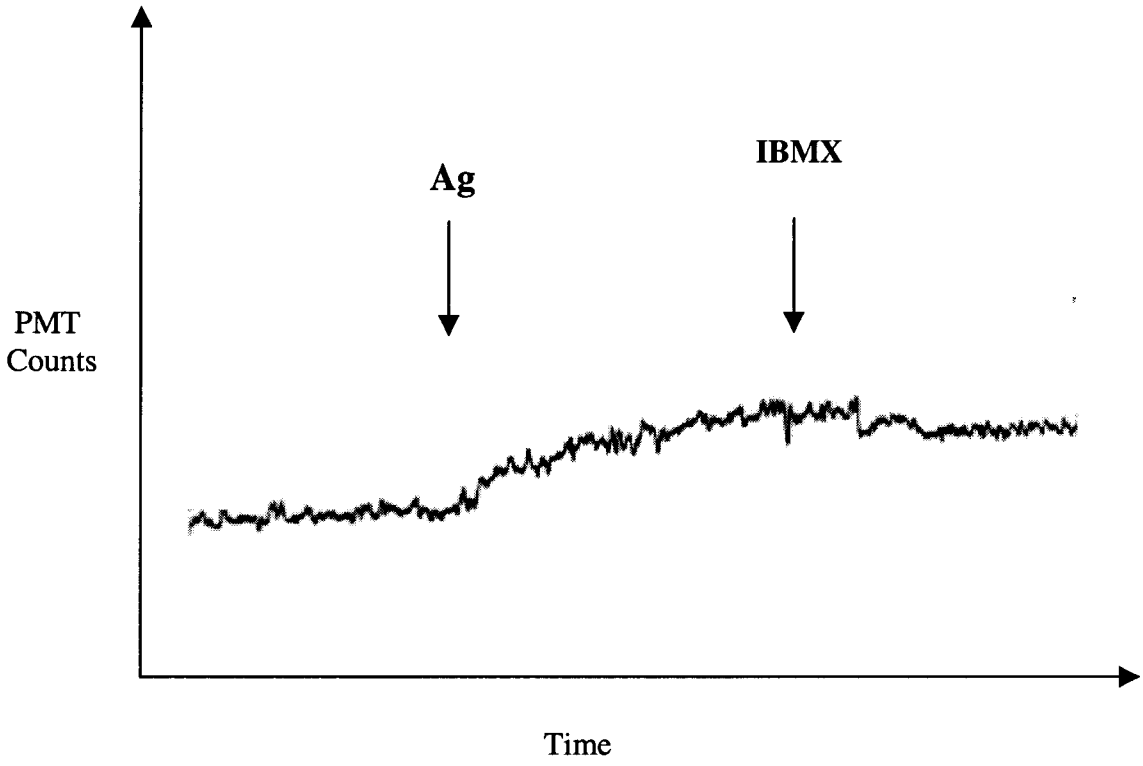


Figure 15: Spectrum of Ag-activated mast cells treated with 1:10dilution of 10 mM IBMX

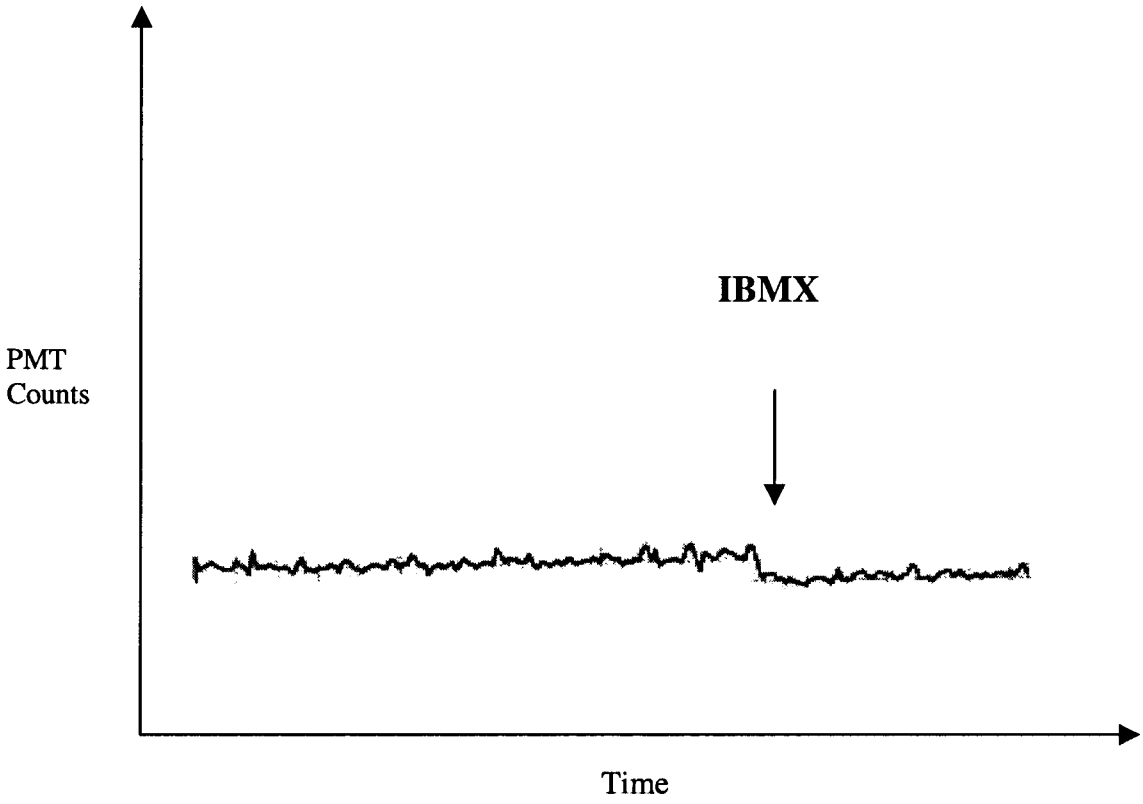


Figure 16: Spectrum of unactivated mast cells treated with 1:10 dilution of 10 mM IBMX

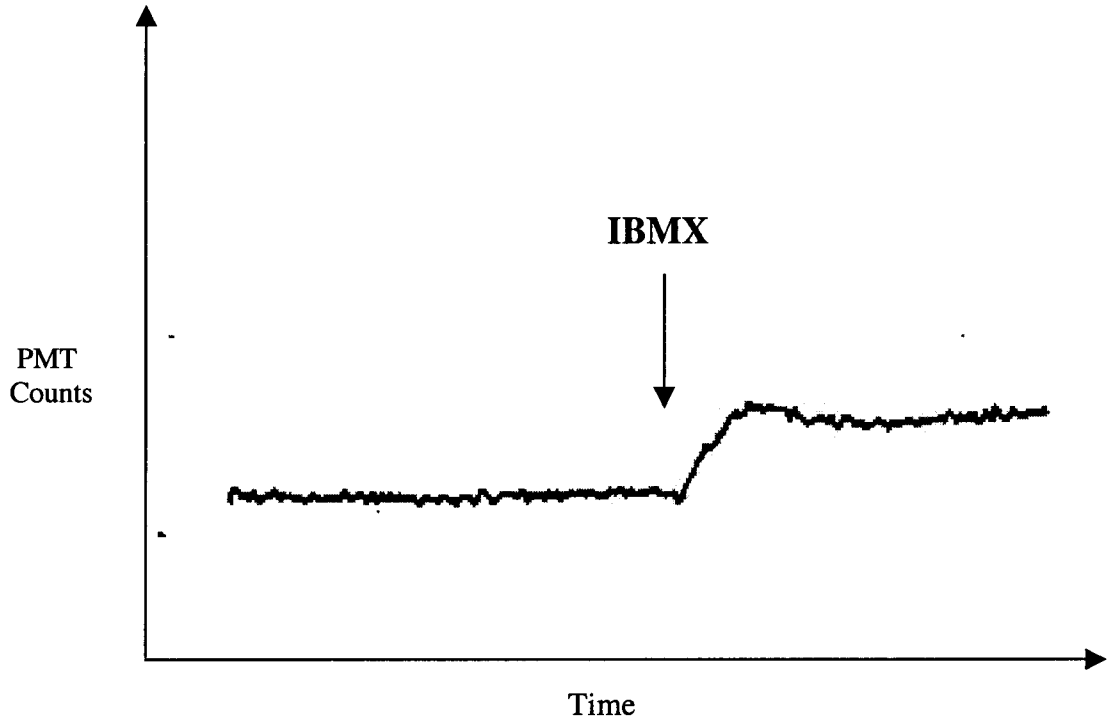


Figure 17: Spectrum of unactivated mast cells treated with of 1 M IBMX

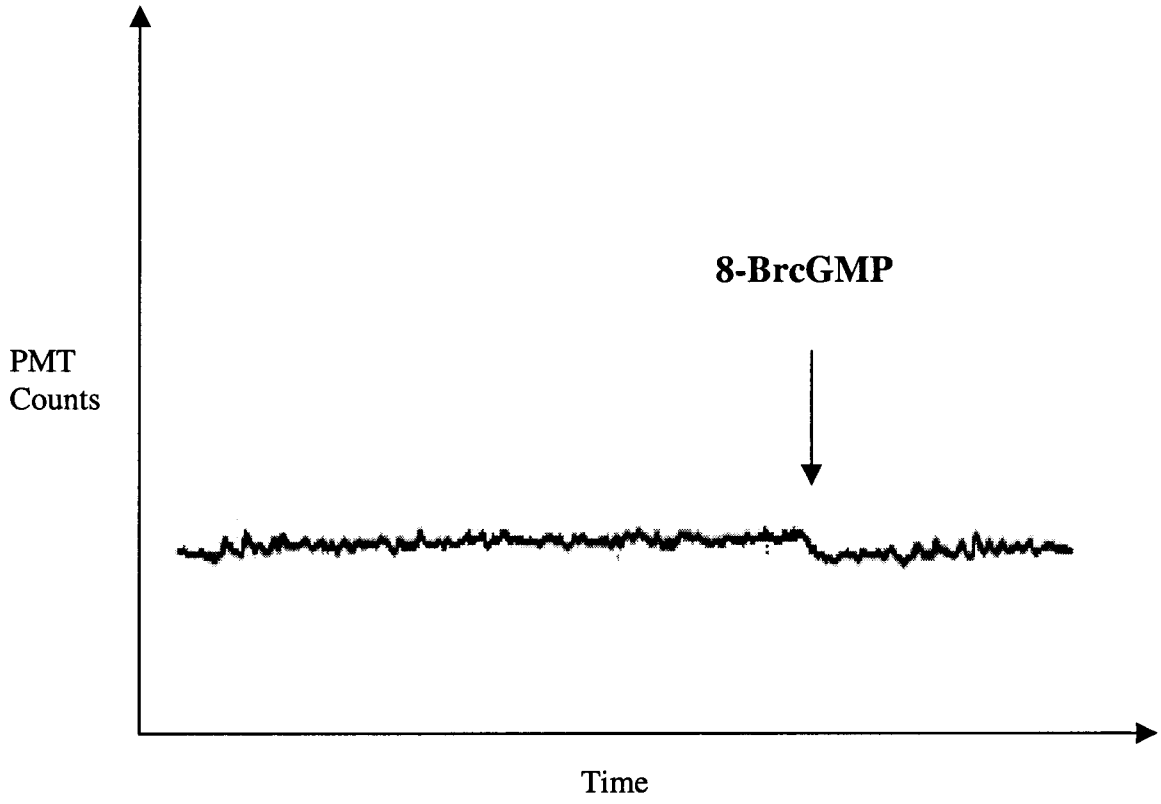


Figure 18: Spectrum of unactivated mast cells treated with 1 mM 8-BrcGMP

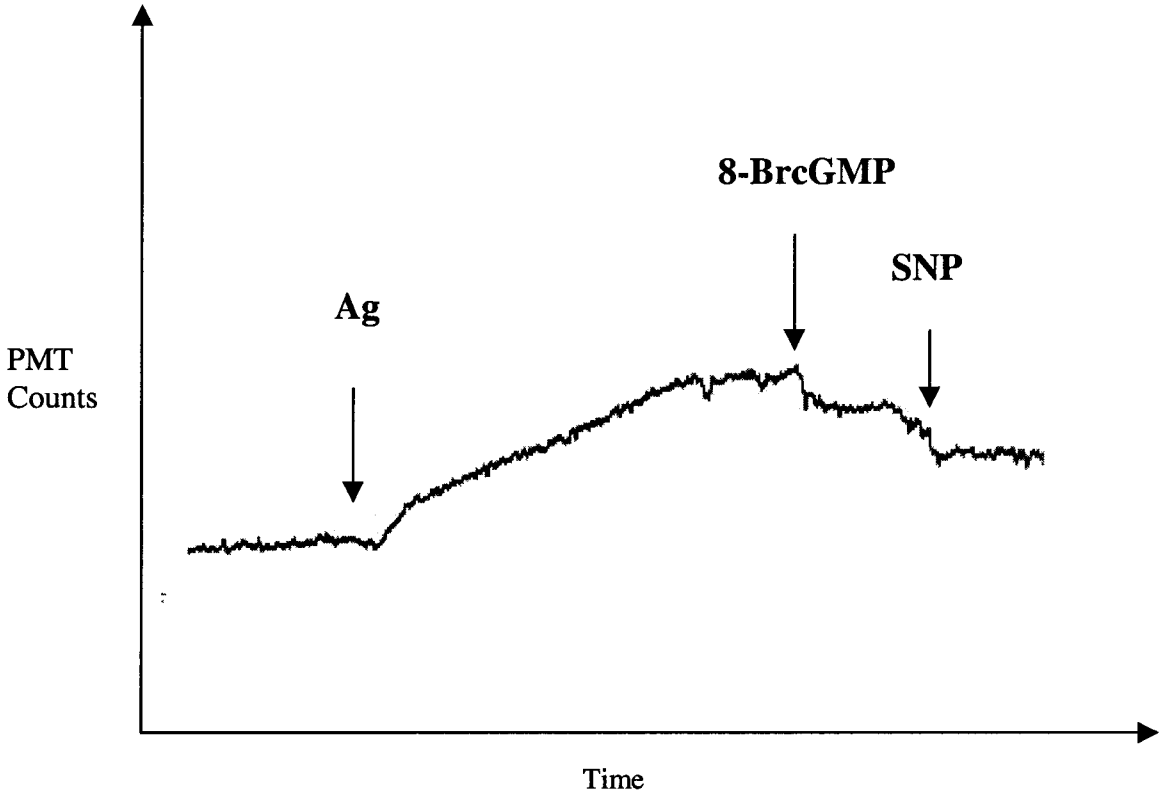


Figure 19: Spectrum of Ag-activated mast cells treated with 1 mM 8-BrcGMP

β-hexoseaminidase Assay

Results from the β-hexoseaminidase assay were used where SNP concentrations (+/-Nac) ranging from 1 x 10^{-3} M to 1 x 10^{-6} M. Trials were run in duplicates and average PMT counts were calculated for each. Standard deviation was accounted for and calculated for each series of runs, represented by the error bars in the histogram. The intensity of the PMT counts is representative of the amount of the fluorescent product. The more product formed is indicative of hydrolyzed substrate and thus more release of β-hexoseaminidase. Nac was used to stabilize the short-lived NO.

The teal-colored bars shown in the Figure 20 represent 0% secretion and 100% secretion. Zero % secretion is representative of unactivated cells and resting levels of β-hexoseaminidase, where 100% secretion represents total β-hexoseaminidase release by cell lysis.

Series 1 (maroon-colored bars) represents assays done using the varying concentrations of SNP in combination with Nac. A continual increase in β-hexoseaminidase secretion was observed as the concentration of SNP (+ Nac) decreased. This correlates to results obtained from the $[Ca^{2+}]_i$ assays and those found in literature that NO modulates $[Ca^{2+}]_i$ by activating the regulatory pathway.

The blue-colored bars in series 2 represents assays done utilizing the varying concentrations of SNP without Nac. A continual increase in β-hexoseaminidase with decreasing SNP concentrations was also seen. As for the individual trials where each SNP concentration was used with and without Nac, a decrease in β-hexoseaminidase secretion was observed with Nac. Concentrations less than 1 x 10^{-6} M SNP were used

which elicited an opposite response in that β-hexoseaminidase decreased with decreasing SNP (+/-) Nac concentrations (data from these trials not shown.).

Secretion was expressed as a percent of the total β- hexoseaminidase content of the cells before stimulation and the results are shown in Figure 21. Percent secretion maintained a continual increase with decreasing SNP concentrations for both + Nac and – Nac trials. For the individual trials of each SNP concentration, there was a decrease in β-hexoseaminidase secretion when Nac was added compared to when Nac was absent. As expected with Nac, NO was stabilized and therefore resided in the cytosol for a longer period of than it normally would.

Average % secretion was graphed and percent error was calculated using standard deviation for each set of SNP concentrations (+/-), represented by the error bars. Percent secretion was calculated according to the following equation:

PMT counts of 100% secretion - PMT counts of 0% secretion = 100% secretion corrected

(PMT counts from trials - PMT counts of 0% secretion) ÷ 100% secretion x 100 = % secretion corrected

From the data, it can be assumed that NO has an inversely proportional effect on $[Ca^{2+}]_i$. This is shown by the trend that occurs in $[Ca^{2+}]_i$ when NO concentrations are varied. It appears that as the concentration of NO reaches insignificant levels, the cells are unable to recognize it and respond. This could be why a decrease in β-hexoseaminidase secretion and thus $[Ca^{2+}]_i$ occurred with SNP concentration below 1 x 10^{-6} M.

Without knowing if contribution of the mast cells is equally distributed or if some are the sole contributors when activated, it is difficult to get a low error when doing more than one trial. The error bars presented in the bar graphs for some of the trials were relatively large, where some had zero error. This could pose a problem and future work looking at individual mast cell response could be an option.

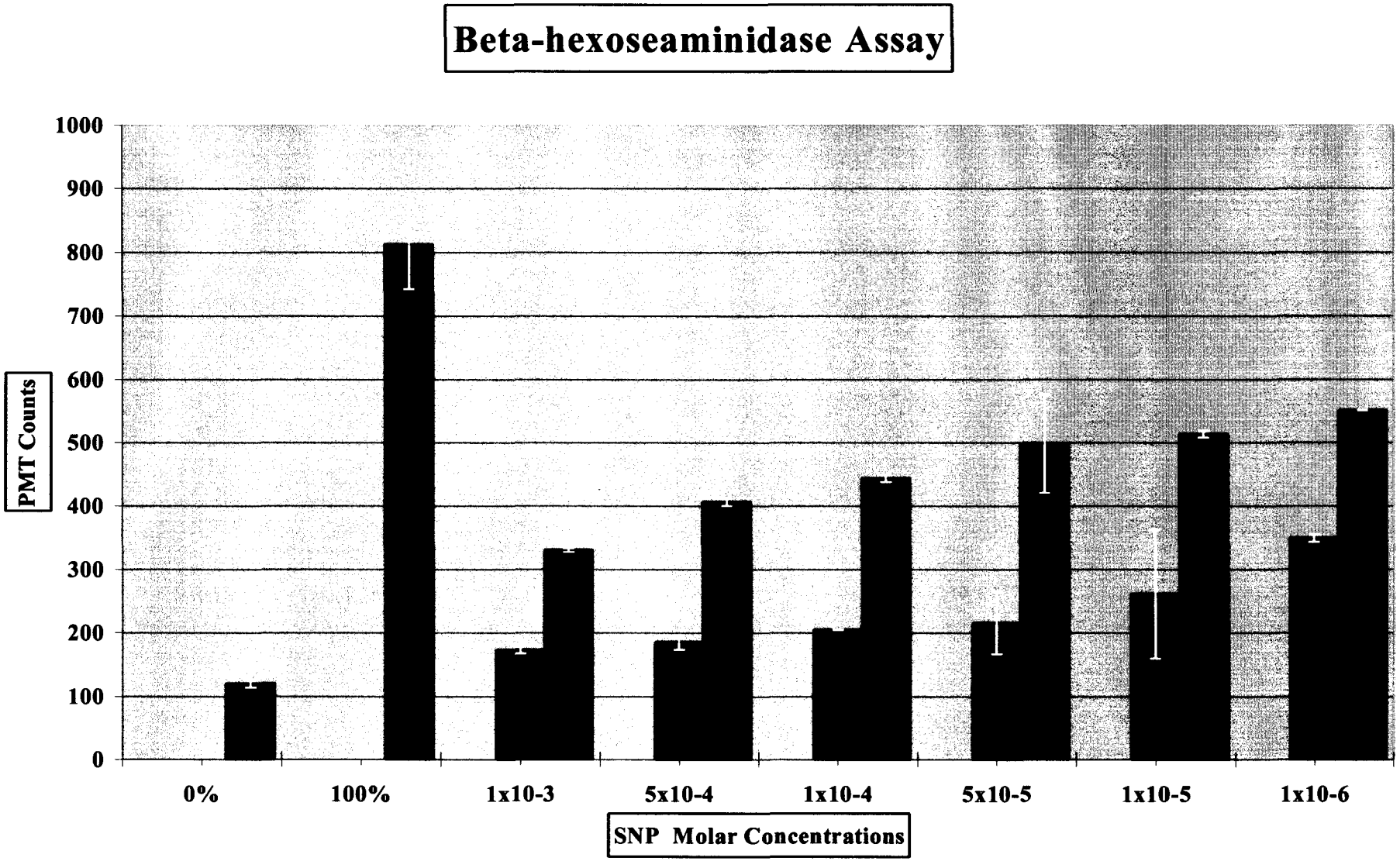


Figure 20: Bar graph representing PMT counts from the β-hexoseaminidase Assay

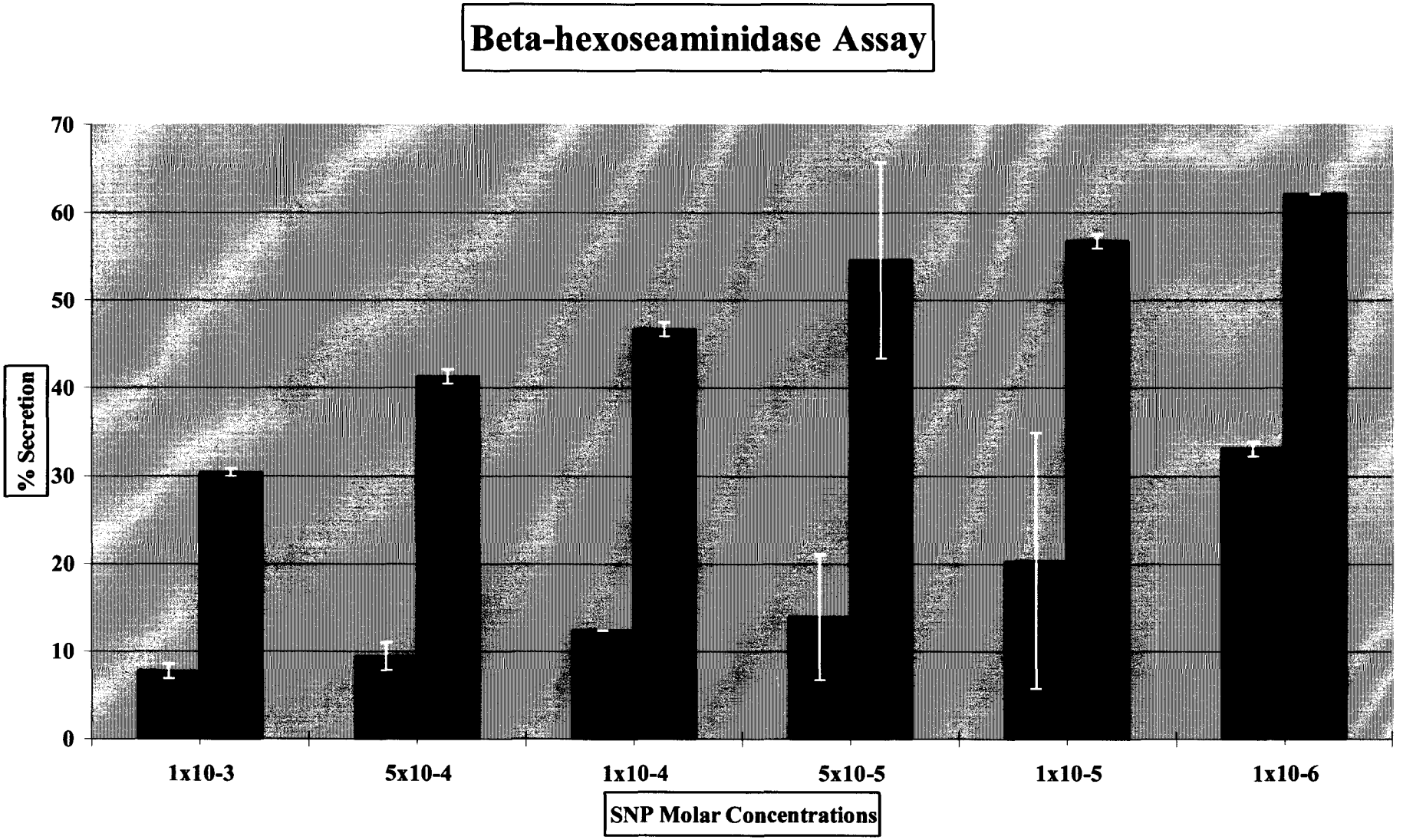


Figure 21: Bar graph representing % secretion from the β-hexoseaminidase Assay

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