STUDIES ON THE STIMULANT ACTION OF HUMAN GAMMA-GLOBULIN ON SPONTANEOUS CONTRACTILITY: INTERACTION WITH K⁺-CHANNEL OPENERS AND PROSTAGLANDIN INHIBITORS

by

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FACULTY OF MEDICINE

We accept this thesis as conforming to the required standard

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Date 28 April, 1993
Abstract

The aim of this thesis was to investigate the stimulatory action of human gamma-globulin on the spontaneous activity of the rat mesenteric portal vein. Previous studies in our laboratory have identified human gamma-globulin and IgG as stimulatory factors which may be responsible for the smooth muscle abnormality associated with the etiology of essential hypertension (Pillai, 1989). This thesis is comprised of three studies. The first study examined whether or not human gamma-globulin exerts its stimulatory action only on spontaneously-active smooth muscles. The second study was to determine if the stimulatory action of human gamma-globulin on the spontaneous activity of the rat mesenteric portal vein is due to decreased potassium conductance. The aim of the third study was to determine if prostaglandins play a role in the stimulatory effects of human gamma-globulin.

Human gamma-globulin significantly increased the contractile activity of spontaneously-active muscles (rat mesenteric portal vein and guinea-pig taenia-caeci) with respect to frequency, force, and integrated response of contraction, whereas it had no significant effect on the contractile activity of quiescent muscles (rat aorta and guinea-pig trachea). At a concentration of 4.35 mg/ml human gamma-globulin caused a 63% increase above the maximum integrated response obtained with the time/volume/pH control in the rat mesenteric portal vein and a 23% increase in
integrated response above that of the time/volume/pH control in guinea-pig taenia-caeci.

Human gamma-globulin had no significant effect on the actions of noradrenaline on the rat mesenteric portal vein. Glibenclamide, a potassium channel antagonist, potentiated the action of human gamma-globulin on the portal vein by 45% and on its own had a biphasic (increase followed by a decrease) effect on the spontaneous activity of the portal vein. Glibenclamide and human gamma-globulin in combination increased the degree of contracture or baseline tone of the portal vein. Diazoxide, a potassium channel opener, non-competitively inhibited the action of human gamma-globulin on the rat mesenteric portal vein by 63%. Both concentrations of pinacidil (0.5 and 5 μM), which is a potassium channel opener, non-competitively inhibited the action of human gamma-globulin by 61% and 78%, respectively. Lemakalim, a potassium channel opener, decreased the spontaneous activity of the portal vein in a concentration-dependent manner. Lemakalim non-competitively antagonized the actions of both noradrenaline and glibenclamide on the rat mesenteric portal vein. Lemakalim potentiated the stimulatory action of human gamma-globulin on the integrated force of the spontaneous contractions of the rat mesenteric portal vein by 40% and 49% at concentrations of 0.5 and 5 μM, respectively. It did so in a manner similar to glibenclamide by interacting with human gamma-globulin to
increase the contracture or baseline tone of the portal vein.

Indomethacin, meclofenamic acid, corticosterone, phenylbutazone, aspirin, ibuprofen, and piroxicam all inhibited the stimulatory action of human gamma-globulin on the rat mesenteric portal vein, but only indomethacin, meclofenamic acid, and corticosterone did so to a significant level. Indomethacin was the most potent inhibitor of human gamma-globulin, decreasing the maximum integrated response of the rat mesenteric portal vein to human gamma-globulin by 40% and 60% at concentrations of $1\times10^{-10}$ M and $1\times10^{-6}$ M. Meclofenamic acid was the second most potent inhibitor of human gamma-globulin, decreasing the maximum integrated response of the rat mesenteric portal vein to human gamma-globulin by 15% and 52% at concentrations of $1\times10^{-10}$ M and $1\times10^{-6}$ M. Corticosterone decreased the maximum integrated response to human gamma-globulin in the rat mesenteric portal vein by 22% at a concentration of $1\times10^{-5}$ M. The order of potency for the remaining NSAIDs was found to be phenylbutazone > aspirin > ibuprofen > piroxicam. In the ex vivo experiment, 10 mg/kg of indomethacin caused a statistically significant decrease in the response of the rat mesenteric portal vein to human gamma-globulin.

It is concluded from these studies that human gamma-globulin exerts its stimulatory effects only on spontaneously active smooth muscle preparations. Findings
from these studies may be taken to suggest that human gamma-globulin, which is a protein, may act by directly modulating a potassium channel such as the maxi-K⁺ channel. It also appears that prostaglandins play a role in the stimulatory action of human gamma-globulin on the rat mesenteric portal vein.
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>$\beta$</td>
<td>beta</td>
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<tr>
<td>BP</td>
<td>blood pressure</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
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<tr>
<td>°C</td>
<td>degree Celsius</td>
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<tr>
<td>g</td>
<td>gram(s)</td>
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<tr>
<td>$&gt;$</td>
<td>greater than</td>
</tr>
<tr>
<td>$\geq$</td>
<td>greater than or equal to</td>
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<tr>
<td>hr(s)</td>
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<tr>
<td>kg</td>
<td>kilogram</td>
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<tr>
<td>MPV</td>
<td>mesenteric portal vein</td>
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<tr>
<td>$\mu$M</td>
<td>micromolar</td>
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<td>M</td>
<td>molar</td>
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<td>mg</td>
<td>milligram</td>
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<td>ml</td>
<td>millilitre</td>
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<tr>
<td>mm Hg</td>
<td>millimetres of mercury</td>
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<td>mM</td>
<td>millimolar</td>
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<tr>
<td>mN</td>
<td>milliNewton</td>
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<tr>
<td>min(s)</td>
<td>minute(s)</td>
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<tr>
<td>NA</td>
<td>noradrenaline</td>
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<tr>
<td>NSAIDs</td>
<td>non-steroidal anti-inflammatory drugs</td>
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<tr>
<td>$\pm$</td>
<td>plus or minus</td>
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<tr>
<td>K$^+$</td>
<td>potassium</td>
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<td>PE</td>
<td>polyethylene</td>
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<td>PG</td>
<td>prostaglandin</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>P.C.O.</td>
<td>potassium channel opener</td>
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<td>pH</td>
<td>hydrogen ion concentration</td>
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<tr>
<td>%</td>
<td>percentage</td>
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<td>s.c.</td>
<td>subcutaneously</td>
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<tr>
<td>sec(s)</td>
<td>second(s)</td>
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<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
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Dedication

This thesis is dedicated to my parents and family.
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1 INTRODUCTION

1.1 Hypertension - an overview

Hypertension can be defined as an elevation of systolic and/or diastolic pressures above 140/90 mm Hg (Gilman et al., 1990). It is estimated that the prevalence of hypertension in Canada is 16% in men and 11% in women using the cutoff point of 140/90 mm Hg (Onrot and Ruedy, 1987). The diastolic pressure is generally used in current classifications of hypertension since increases observed in diastolic pressure tend to be smaller and more consistent compared with changes in the mean systolic pressure, which increases non-linearly with age (Hamilton, 1954; Gordon, 1964). The etiology of up to 90% of all hypertension is unknown and thus classified as "essential" or "primary" hypertension (de Champlain, 1978). The term "essential" as applied to hypertension was based on the mistaken impression that blood pressure elevation was essential to push blood through vessels narrowed by age (Katzung, 1989). The remaining 10% has an identifiable origin and is classified as "secondary" hypertension.

The diagnosis of hypertension is based on repeated, reproducible measurements of elevated blood pressure (Campbell et al., 1990). Hypertension is typically classified as mild (90-104 mm Hg), moderate (105-114 mm Hg), or severe (>115 mm Hg) depending on the level of the diastolic blood pressure (Andreoli et al., 1990). Hypertension is not a disease and it is usually asymptomatic.
The major health consequences of hypertension are its attendant risk for cardiovascular, cerebrovascular, and renal complications (Kannel, 1977). The risks of elevated blood pressure have been determined by numerous large scale epidemiological studies (Kannel and Sorhe, 1975; Pooling Project Research Group, 1978; Spence et al., 1980). These studies and other studies (Paul, 1971; Helgeland, 1980) indicate a positive correlation between elevated blood pressure and increased morbidity and mortality, with the increased risk closely paralleling the degree of diastolic blood pressure elevation.

Clinical trials have shown that appropriate pharmacological treatment of hypertension significantly reduces the risk of stroke, renal failure and congestive heart failure associated with high blood pressure in patients with moderate to severe hypertension (Helgeland, 1980; Amery et al., 1985; MacMahon et al., 1986; Frohlich et al., 1988). Despite numerous studies, there is still no distinct dividing line between normal and pathological blood pressures. Many large scale clinical trials (Veterans Administration Cooperative Study, 1970; U.S. Public Health Service Hospital Cooperative Study Group, 1977; Helgeland, 1980) have addressed the question of whether or not to treat patients with modest elevations of blood pressure (for review see Shackleton and Ruedy, 1984). It is clear from these and other studies that antihypertensive drug therapy
benefits all patients with diastolic pressures > 95 mm Hg, but it is still not clear whether treatment of patients with diastolic pressures of 90 to 94 mm Hg is beneficial (Robertson, 1987), despite the fact that these people are at a higher risk of developing cardiovascular disease than are individuals with normal blood pressure (Gilman et al., 1990). Although the benefits of antihypertensive drug therapy for patients with borderline or mild hypertension is still controversial, most studies suggest that non-pharmacological treatment may be valuable for this group of people (Shackleton and Ruedy, 1984). Patients with persistent diastolic pressures between 90 and 94 mm Hg require individualized treatment, but generally should be advised regarding lifestyle modifications, such as stopping smoking, exercise, and weight reduction in the obese.

Essential hypertension is not a discrete entity, but rather a heterogeneous syndrome in which multiple factors may contribute to the elevated blood pressure (Katzung, 1989). When diagnosing hypertension, it is important to consider not only the level of the blood pressure, but also: age, sex, race, smoking, family history, obesity, glucose intolerance, and high LDL- and low HDL- cholesterol (Williams, 1991). It has been determined by large scale epidemiological surveys (Kannel and Sorhe, 1975; Pooling Project Research Group, 1978; Spence et al., 1980) that the prevalence and risks of hypertension vary among race, sex, and age groups. The risk of hypertension tends to increase
with advancing age. In the United States, urban blacks have twice the prevalence rate for hypertension as whites and more than four times the hypertension-associated morbidity rate (Williams, 1991). Women generally have a lower prevalence of hypertension than men (Onrot and Ruedy, 1987). There is clearly a positive correlation between obesity and arterial pressure (Andrews et al., 1982). Weight gain is associated with an increased incidence of hypertension in normotensive subjects and weight loss in obese subjects with hypertension has been shown to lower their arterial pressure (Fletcher et al., 1988; Williams, 1991). Accelerated atherosclerosis is a companion of hypertension and thus, it is not surprising that independent risk factors associated with the development of atherosclerosis (such as elevated serum cholesterol, glucose intolerance, and/or smoking) significantly enhance the effect of hypertension on mortality rates regardless of age, sex, or race (Onrot and Ruedy, 1987; Bierman, 1991). Epidemiologic evidence also suggests that genetic inheritance (Havlik and Feinleib, 1982; Longini et al., 1984), psychologic stress (Katzung, 1989), as well as environmental and dietary factors (increased salt and decreased calcium intake) (Beard et al., 1982; MacGregor et al., 1982; Onrot and Ruedy, 1987; Williams, 1991) may contribute to the development of hypertension. It has been reported that an increase in blood pressure with aging does not occur in populations with low sodium intake (Katzung, 1989).
1.2 Changes in vascular smooth muscle associated with hypertension

Although the etiology of essential hypertension has been extensively investigated for the last several decades, no single causative factor has been identified. It is, however, generally accepted that the primary abnormality in human essential hypertension is the increase in the peripheral resistance (Kaplan, 1986). The contractile state of vascular smooth muscle is altered in essential hypertension, this being important since vascular smooth muscle is the regulator of total peripheral resistance and its contractile state determines the arterial blood flow. The cause of this vascular smooth muscle abnormality is not known and it is still not clear whether the primary abnormality giving rise to the increased peripheral resistance relates to structural or functional changes in the vascular smooth muscle (Spray and Roberts, 1977; Curtis and Seehar, 1978; Winquist and Bohr, 1983; Laher and Triggle, 1984; Slack et al., 1984; Pang and Scott, 1985; Aalkjer et al., 1987). There is evidence showing that the sensitivity of vascular smooth muscle to calcium is altered in essential hypertension (Sutter et al., 1977; Fitzpatrick and Szentivagi, 1980; Devynck et al., 1981; Lipe and Moulds, 1985), but not much direct evidence to show that the hypertensive vascular smooth muscle per se handles calcium differently (Harris et al., 1984; Sutter, 1985). The only
direct evidence for increased membrane permeability to calcium in hypertension came from studies on erythrocytes (Devynck et al., 1981; Chan et al., 1983).

1.3 Possible role of circulating plasma or serum factors in hypertension

Recently it has been suggested that the immune system may play a role in the etiology of essential hypertension (for reviews see Khraibi, 1991; Dzielak, 1992). A study conducted by Ebringer and Doyle (1970) showed a positive correlation between raised serum IgG levels and essential hypertension. This observation has been confirmed by other researchers (Olsen et al., 1973; Kristensen, 1978; Gudbrandsson et al., 1981; Kristensen and Solling, 1983). The raised IgG levels persist in spite of lowering blood pressure; thus, pressure per se is not likely responsible for the increase in IgG concentrations (Kristensen, 1978).

Previous reports in the literature have shown that serum or plasma from hypertensive animals sensitize vascular tissue to pressor agents (Michelakis et al., 1975; Wright, 1981; Cappuccio et al., 1986). Other reports have shown that the administration to experimental animals of a low molecular weight protein obtained from hypertensive human urine induces hypertension (Sen et al., 1977). Greenberg et al. (1975) showed that the administration to animals of hypertensive serum from humans enhanced the pressor responses of the recipient animals to vasoactive substance such as noradrenaline and tyramine. Wright and McCumbee...
(1984) and McCumbee and Wright (1985) have demonstrated that a small molecular weight peptide extracted from red blood cells of hypertensive rats had a stimulatory effect on calcium uptake by tissues in vitro and a hypertensive effect when injected into normotensive rats in vivo. Lindner et al. (1987) reported that when platelets from normotensive patients were incubated with plasma from hypertensive patients the cytosolic free calcium increased.

A previous report from our laboratory showed that plasma from hypertensive patients had a concentration-dependent biphasic excitatory and inhibitory effect on the spontaneous contractile activity of the rat mesenteric portal vein in vitro (Pillai and Sutter, 1989). The spontaneous activity of the rat mesenteric portal vein at any given concentration of hypertensive plasma was significantly higher than that of normotensive plasma. These observations are consistent with the presence of both excitatory and inhibitory substances in the plasma and may be taken to imply that plasma from the hypertensive patients contains more of the excitatory substances or less of the inhibitory substances. Other studies by our laboratory examined the effects of several human plasma proteins on the spontaneous contractility of the rat mesenteric portal vein and found that albumin and gamma-globulin stimulated, whereas alpha- and beta-globulin inhibited spontaneous contractions (Pillai and Sutter, 1990). Albumin (55-65% of the total plasma proteins), gamma-globulin (17-27% of the
total plasma proteins), alpha-globulin (14-18% of the total plasma proteins), and beta-globulin (14-18% of the total plasma proteins) are the major plasma proteins present in the plasma and IgG (75% of the total immunoglobulin) is the major immunoglobulin present in the gamma-globulin fraction (Burton and Gregory, 1986). The effect of Albumin was found to be adrenomimetic while gamma-globulin stimulated vasomotion by a non-adrenergic, non-cholinergic action which did not occur in the absence of an electrically excitable membrane.

1.4 The rat mesenteric portal vein as a model for resistance vessels

Blood pressure is maintained by the action of the heart and the resistance to blood flow in the blood vessels. It is clear that an abnormality in the vasculature plays a role in hypertension since the heart is normal in early hypertension. Increased resistance to blood flow results in increased blood pressure. Resistance to flow increases as the diameter of the blood vessels decreases. The diameter of blood vessels is determined by the degree of contraction of the smooth muscle in their walls as well as by their physical structure. Thus, vascular smooth muscle plays a role in hypertension. The blood vessels responsible for the maintenance of blood pressure are the arterioles or resistance vessels, but these vessels are too small to study directly. Thus, one must use either arteries or veins. Large arteries are not only dissimilar to
arterioles, but are also exposed to high pressure which makes it difficult to determine whether changes in their structure cause high blood pressure or are a result of high blood pressure. We use the rat mesenteric portal vein, which drains blood from the gut to the liver, as a model for resistance vessels since unlike other veins it contains an abundance of smooth muscle and unlike the arteries, veins are not exposed to high pressure. Since the bulk of the smooth muscle in the portal vein is oriented longitudinally (Sutter, 1965), longitudinal strips or whole veins mounted longitudinally are the usual preparation.

The rat mesenteric portal vein has been used extensively during the past 25 years as a model for resistance vessels (for review see Sutter, 1990) because physiologically it resembles resistance vessels. The similarities between the rat mesenteric portal vein and the resistance vessels include a high ratio of muscular to elastic tissue, the presence of action potentials and vasomotion, and the dependence on the presence of external calcium to sustain responsiveness (Cuthbert et al., 1964; Axelsson et al., 1967; Ljung, 1970; Rhodes and Sutter, 1971; Collins et al., 1972; Sutter et al., 1977). Additionally, the rat mesenteric portal vein was used as a screening model of resistance vessels in the development of the drug felodipine, a vascular selective calcium channel antagonist, which has marked effects on the peripheral resistance and is an effective anti-hypertensive agent. Agents which
increase the spontaneous contractility of the rat mesenteric portal vein are likely to also increase the contractile activity of the peripheral resistance vessels and consequently increase the peripheral resistance.

1.5 Objectives

Previous studies in this laboratory have identified human gamma-globulin as a substance which increases the spontaneous activity of the rat mesenteric portal vein (Pillai and Sutter, 1990). It has also been shown by our laboratory that prior treatment of the rat mesenteric portal vein with ouabain or the calcium channel antagonist verapamil prevents the stimulatory effects of human gamma-globulin on the rat mesenteric portal vein (Pillai and Sutter, 1990). Ouabain abolishes the spontaneous activity of the mesenteric portal vein, but it does not prevent the contraction of the mesenteric portal vein to noradrenaline (Matthews and Sutter, 1967). It was also observed that the stimulatory effects of human gamma-globulin on the spontaneous contractility of the rat mesenteric portal vein are not blocked by the antagonists of common neurotransmitters such as noradrenaline, acetylcholine, histamine, serotonin, and angiotensin II (Pillai and Sutter, 1990). These findings suggest that receptors to the common neurotransmitters are not involved in the excitatory effects of human gamma-globulin, but that a polarised and/or spontaneously depolarising membrane is necessary for smooth muscles to respond to gamma-globulins.
Smooth muscles vary in the nature of their membranes: some, such as the rat mesenteric portal vein and guinea-pig taenia-caeci, are spontaneously active and demonstrate action potentials, while others such as the rat aorta and guinea-pig trachea are not spontaneously active and do not show action potentials. Previously, these were the criteria used by Bozler (1941, 1948) to classify smooth muscles as either multi-unit (quiescent) or unitary (spontaneously active). The aim of the first part of this thesis was to determine whether or not human gamma-globulin exerts its stimulatory effect only on smooth muscles with spontaneous activity. Human gamma-globulin concentration-response curves were constructed on the following tissue preparations: the rat mesenteric portal vein (spontaneously active), rat aortic rings with and without intact endothelium (quiescent), the guinea-pig taenia-caeci (spontaneously active), and guinea-pig tracheal chains (quiescent).

Previous studies have shown that the rat mesenteric portal vein can be stimulated by a variety of different agents, including local anaesthetic agents such as procaine (Sanders, 1969). There is evidence that procaine decreases potassium conductance in the rat mesenteric portal vein (Hara, 1980) and that this could be involved in the excitant effects of procaine on this tissue. The contractile effects of procaine, like those of human gamma-globulin, are prevented by ouabain treatment (Sanders, 1969). This
observation suggests that decreased potassium conductance is common to the effects of both procaine and human gamma-globulin and mediates the excitant effects of each on smooth muscle. The aim of the second part of this thesis was to examine whether human gamma-globulin stimulates spontaneous contractility in the rat mesenteric portal vein by inactivating potassium channels. In order to do this, a novel group of antihypertensive agents that were recently developed and which have been classified as potassium channel activators or potassium channel openers (PCOs) (Cook et al., 1988; Triggle et al., 1992) were used. Gamma-globulin concentration-response curves were examined in the presence of glibenclamide (glybenclamide, glyburide), an antagonist of ATP-sensitive potassium channels (Standen et al., 1989; Winquist et al., 1989), and the potassium channel activators diazoxide, pinacidil, and lemakalim (BRL 38227) (Winquist et al., 1989; Shen et al., 1991).

It has been suggested that prostaglandins may be related to the spontaneous activity and tone in vessels and other tissues (Daniel and Sarna, 1978). Enero (1979) reported that the prostaglandin inhibitor sodium meclofenamate depressed the spontaneous contraction of the rat mesenteric portal vein by about 50% without modifying the responses to noradrenaline. Thus, the possibility exists that agents such as prostaglandins, leukotrienes, thromboxanes, and platelet-activating factor are involved in the stimulatory response to human gamma-globulin that was
reported by our laboratory. In the third part of this thesis we tested this possibility, by examining the effects of various non-steroidal analgesic and anti-inflammatory drugs (NSAIDs) on the stimulatory action of human gamma-globulin in the rat mesenteric portal vein. Human gamma-globulin concentration-response curves were constructed in the presence of: aspirin, ibuprofen, indomethacin, meclofenamic acid, piroxicam, phenylbutazone, and corticosterone. Additionally, an ex vivo experiment with indomethacin was done.
2. MATERIALS AND METHODS
2.1 In vitro experiments
2.1.1 Tissue preparations
2.1.1.1 Preparation of rat mesenteric portal vein

Male Wistar rats (300-400 g) were stunned by a blow to the head and killed by cervical dislocation followed by exsanguination. The abdominal cavity was opened, and the mesenteric portal vein was separated from the connective tissue using blunt dissection techniques as described by Pang and Sutter (1981). A thread was tied to the distal end of the vein leaving enough thread so that later it could be tied directly to the tissue holder. At the proximal end of the vein a long thread was attached to connect to the Grass FT-03-C force-displacement transducer. Before removing the mesenteric portal vein from the rat, a small slit was made in one side of the vein to allow blood to drain. The portal vein was then mounted for isometric recording from the force-displacement transducers at a passive force of 5 mN and allowed an equilibration period of 1 hour before experiments were carried out.

2.1.1.2. Preparation of rat aortic rings

The thoracic aorta was removed from male Wistar rats (250-350 g) and cleared of connective tissue; care was taken to protect the endothelial lining from being damaged. The aorta was cut into 2.5 mm wide transverse rings and mounted under 1 g resting tension on stainless steel hooks. Rings were allowed to equilibrate for 60 minutes before the
experiments were begun. Endothelial cells were removed from some aortic rings by gently rubbing the intimal surface with a wooden stick for 30 seconds.

2.1.1.3. Preparation of guinea-pig tracheal ring chains

This preparation of the guinea-pig tracheal chains was based on the method of Castillo and De Beer (1947). Briefly, male guinea-pigs (300-350 g) were killed by a blow to the head. The neck and upper thorax were opened up and the muscles surrounding the trachea were cleared by blunt dissection. Approximately 6 cm of trachea was dissected out and transferred to a Petri dish containing Krebs solution. At least six rings of muscle were cut from the trachea by making transverse cuts. Each ring contained two bands of cartilage. The rings were D-shaped and the smooth muscle was found on the straight part of the D. The rings were tied together with surgical thread attached to the cartilage so that the smooth muscle was in a longitudinal plane with each alternate ring having smooth muscle on the opposite side. Each tracheal chain preparation consisted of three rings which were mounted in an organ bath and allowed to equilibrate for 45 minutes.

2.1.1.4 Preparation of guinea-pig taenia-caeci

This preparation of the guinea-pig taenia-caeci was based on the method of Burnstock et al. (1965). Briefly, the abdominal cavity of the guinea-pig was opened up and the caecum was located. The taenia which lies on the surface of the caecum was dissected out and trimmed of any connective
tissue. A segment 3-4 cm was then mounted in an organ bath for measurement of mechanical response under a passive force of 10 mN and allowed an equilibration period of 60 minutes.

2.1.2 Experimental protocol

Following the equilibration period, experiments were carried out (minimum of n=6 in each set of experiments). All experiments were performed at 37°C in either 5 ml or 20 ml organ baths filled with Krebs solution bubbled with 95% O₂ and 5% CO₂ (carbogen). The Krebs solution had the following composition (mM): NaCl, 112; KCl, 4.5; CaCl₂, 2.5; KH₂PO₄, 1.2; NaHCO₃, 2.5; glucose, 11.1; EDTA, 0.026; MgCl₂·6H₂O, 1.2. The experimental procedure has been described previously (Pillai and Sutter, 1990). Briefly, the pH of the Krebs solution was adjusted to 7.6 so that it matched the pH of the plasma protein solution. Since the bubbling of plasma protein solution with carbogen produced foaming, bubbling of the bathing solution was stopped when plasma proteins were added. A pH/time/volume control was done in a manner identical to test conditions, including stopping of bubbling, to ensure that any effects observed on the smooth muscle tone were not due to either a change in volume, pH, bubbling, or any combination of these factors. A magnetic stirrer was used to stir the bath solution after each addition of a drug or solution. Human gamma-globulin concentrations were altered by serial addition of Krebs containing dissolved drug as described by Pillai and Sutter (1990). The pH of the bath solution was monitored during
the experiments with either litmus paper or phenol red indicator.

Tissues with spontaneous activity were allowed 10 to 15 minutes to stabilize after the addition of the test drug or solution before a concentration response curve to human gamma-globulin or noradrenaline was constructed. The four minutes immediately preceding the gamma-globulin curve were used as the baseline value. The maximum integrated response of each preparation was set at 100% and all other responses were expressed as a percentage of this value. Thus, each tissue served as its own control and all values were normalized relative to the maximum response demonstrated by each individual tissue preparation. In all cases, contractile activity was measured for 4 minutes after the addition of a drug or solution. All tissues were washed repeatedly during the equilibration period and between curves. Tissues were allowed to recover in Krebs solution bubbled with carbogen between curves.

2.1.3 Measurement of contractile activity

All tissue preparations were connected to Grass FT-03-C force-displacement transducers for isometric recording. The transducer signals were amplified and recorded on a Grass polygraph (model 7). The amplified signals from the portal vein and taenia-caeci (tissue preparations with spontaneous activity) were integrated electronically using a Grass integrator (model 7 P10 B) over 1 minute intervals on a separate channel and displayed on the polygraph as force-
time (integrated) response as well as real-time responses (Pang and Sutter, 1980). The spontaneous contractile activity was then calculated as frequency (number of spikes/minute), force (mean tension in grams), and integrated response (grams·minutes).

2.2 Ex vivo experiments

2.2.1 Surgical preparation of rats

Male Wistar rats (300-400 g) were anaesthetized with halothane (4% in air for induction, 1.5% in air for maintenance). A polyethylene cannula (PE 50) was inserted into the left iliac artery for the measurement of arterial pressure by a pressure transducer (P23 D B, Gould Statham, Oxnard, CA, U.S.A.). PE 50 cannulae were also inserted into both iliac veins for the administration of drugs. All cannulae were filled with heparinized normal saline (25 I.U./ml) and tunneled subcutaneously along the back, exteriorized and secured at the back of the neck. The rats were allowed to recover from surgery and the effects of halothane for 24 hours before experiments were started.

2.2.2 Experimental protocol

All experiments were carried out on conscious rats one day after surgery. Indomethacin (10 mg/kg) or the equivalent volume of vehicle were infused as bolus injections at a rate of 0.08 ml/min/kg over 5 minutes. The rats were killed one hour after the bolus infusion of either indomethacin or vehicle and the portal vein was mounted in a 20 ml tissue bath as described above in section 2.1.1.1.
Human gamma-globulin concentration-response curves were constructed as described in section 2.1.2. after an equilibration period of 1 hour. Data are expressed as percentage change from baseline.

2.3 Drugs

Human gamma-globulin, indomethacin, phenylbutazone, aspirin, corticosterone, ibuprofen, piroxicam, meclofenamic acid, noradrenaline, histamine, and acetylcholine were all obtained from Sigma Chemical Co., St. Louis, MO, USA. Human gamma-globulin was dissolved in Krebs solution a few minutes before use as done by Pillai and Sutter (1990). Pinacidil (Eli Lilly and Co., Indianapolis, Indiana, USA), lemakalim (SmithKline-Beecham Pharmaceuticals, England) glibenclamide (Glyburide Micronized, Hoechst, Quebec, Canada), aspirin, and meclofenamic acid were all dissolved in double distilled demineralized water. For the concentration-response curve, glibenclamide was dissolved in 95% dimethyl sulfoxide (DMSO) (Sigma Chemical Co, St. Louise, MO, USA). Diazoxide for intravenous use (Schering Canada Inc., Quebec) was diluted using double distilled demineralized water. Noradrenaline (Sigma Chemical Co., St. Louise, MO, USA) was dissolved in 0.01 N HCl. Indomethacin, corticosterone, phenylbutazone, ibuprofen, and piroxicam were all dissolved in 80% ethanol and diluted 1000 fold to give a vehicle bath concentration of 0.08% ethanol. All noradrenaline, histamine, acetylcholine, indomethacin, meclofenamic acid, ibuprofen, corticosterone, phenylbutazone, piroxicam, and aspirin
stock solutions were made up fresh daily and serial dilutions were done using double distilled demineralized water.

2.4 Statistical analysis

All data were analysed by the Analysis of Variance (ANOVA) statistical test which is used when treatments are done under uniform conditions (Li, 1964) and allows for the comparison of many treatments (Zar, 1984). Following the ANOVA test, a post hoc or multiple comparison test was used for the comparison of group means. Duncan's multiple range test (Duncan, 1955) compares groups of continuous and randomly distributed data of equal sample size and was chosen since it is one of the most powerful tests available for detecting differences between means (Montgomery, 1984). A probability error of p<0.05 was pre-selected as the criterion for statistical significance. The maximum integrated response of each preparation was set at 100% and all other responses were expressed as a percentage of this value. Results are expressed as means ± S.E.M. and a spline function was used to fit the curves.
3. RESULTS

3.1 Stimulant effect of human gamma-globulin on smooth muscle preparations

3.1.1 In vitro effects of human gamma-globulin on spontaneously active muscle preparations

Human gamma-globulin caused a concentration-dependent increase in both the amplitude and frequency of the spontaneous activity of both the rat mesenteric portal vein and guinea-pig taenia-caeci. The maximum increase in integrated response was 3 to 4 fold above that of the pH/time/volume controls for the rat mesenteric portal vein (Fig. 1) and the guinea-pig taenia-caeci (Fig. 2). Gamma-globulin exerted its maximum effect on the integrated response in both these preparations at a bath concentration of 4.35 mg/mL. A histamine concentration-response control curve (3x10^{-9} M to 3x10^{-4} M) on the guinea-pig taenia-caeci was constructed and is shown in figure 2.

3.1.2 In vitro effects of human gamma-globulin on quiescent smooth muscle preparations

The aortic rings (with and without endothelium) were precontracted with phenylephrine and then an acetylcholine relaxation curve was constructed to confirm whether or not the endothelium was still intact. Human gamma-globulin did not significantly alter the contractile activity of the rat aortic rings (either with or without endothelium) compared to the time/volume controls as shown in figures 3 and 4. These aortic rings did contract appropriately to
noradrenaline (10^{-9} \text{ M} \text{ to} 10^{-4} \text{ M}) as shown in figures 3 and 4. Human gamma-globulin did not significantly contract the guinea-pig tracheal chains in comparison to the volume/time control curve (Fig. 5). However, histamine (3 \times 10^{-9} \text{ M} \text{ to} 3 \times 10^{-4} \text{ M}) caused a concentration-related contracture of the tracheal chains (Fig. 5).

3.2 *In vitro* effect of human gamma-globulin on the action of noradrenaline in the rat MPV

Figure 6 shows that the maximum force of contraction developed by the portal vein to noradrenaline tended to decrease in the presence of human gamma-globulin, but statistically there was no significant difference between the maximum integrated force of contraction developed to noradrenaline in the absence (1.01 \pm 0.04 \text{ grams\cdot min}) or presence (0.86 \pm 0.06 \text{ grams\cdot min}) of human gamma-globulin (Table 3). The frequency (Table 1) and force or amplitude (Table 2) of the spontaneous activity of the portal vein were also decreased in response to noradrenaline in the presence of gamma-globulin, but not to a statistically significant level.

3.3 *In vitro* effect of a potassium channel blocker on the action of human gamma-globulin in the rat MPV

Figure 7 shows that glibenclamide (5 \mu\text{M}) increased the stimulant effect of human gamma-globulin on the spontaneous contractions of the rat MPV in a concentration-dependent manner. Glibenclamide increased the maximum integrated contractile response developed by the portal vein to gamma-
globulin by 45% above that of the gamma-globulin control (Fig. 7). The frequency (Table 1), force (Table 2), and integrated response (Table 3) of the spontaneous contractions of the rat MPV as well as the contracture or tone of the rat MPV (Fig. 13) were all significantly increased above control values by glibenclamide (5 μM) + gamma-globulin. A time control for glibenclamide (5 μM) was done and the results are also shown in figure 7. Glibenclamide itself had a biphasic action on the spontaneous activity of the rat MPV, first increasing and then decreasing the frequency, force, and integrated response of the spontaneous contractions of the portal vein.

3.4 *In vitro effects of potassium channel activators or openers on the action of human gamma-globulin in the rat MPV*

Figure 8 shows that diazoxide (5 μM) insurmountably blocked the stimulatory action of human gamma-globulin on the spontaneous activity of the rat MPV, which resulted in a decreased maximum response that could not be recovered by increasing concentrations of human gamma-globulin. Diazoxide (5 μM) decreased the maximum integrated response of the portal vein to human gamma-globulin by 75% (Fig. 8). Diazoxide also decreased the frequency (Table 1), force (Table 2), and integrated response (Table 3) of the spontaneous contractions of the portal vein in the presence of human gamma-globulin.
Figure 9 shows that pinacidil (0.5 and 5 µM) insurmountably blocked the stimulatory action of human gamma-globulin on the spontaneous activity of the rat MPV in a concentration-dependent manner. The lower concentration of pinacidil (0.5 µM) decreased the maximum integrated response of the portal vein to human gamma-globulin by 61% and the higher concentration of pinacidil (5 µM) decreased the integrated response to human gamma-globulin by 78% (Fig. 9). Both concentrations of pinacidil (0.5 and 5 µM) lowered the response of the portal vein to human gamma-globulin with respect to frequency (Table 1), force (Table 2), and integrated response (Table 3).

Figure 10 shows that lemakalim (0.5 and 5 µM) tended to increase, rather than inhibit, the stimulant action of human gamma-globulin on the maximum integrated response of the spontaneous contractions of the rat MPV in a concentration-dependent manner. Table 2 shows that lemakalim decreased the force of contractions produced by human gamma-globulin; however, Table 3 shows that lemakalim actually increased the integrated response of the rat mesenteric portal vein to human gamma-globulin. The effect of lemakalim on the action of human gamma-globulin on the frequency of the spontaneous contractions of the rat MPV could not be determined since lemakalim + human gamma-globulin produced a transient increase in the number of spikes/minute for approximately 4 to 8 minutes or for the first two concentrations of human gamma-globulin followed by a cessation in spiking frequency
(Figs. 13 B and 13 C). The effects which were observed with lemakalim plus gamma-globulin on the degree of contracture and frequency of spontaneous activity of the portal vein were similar to those produced by glibenclamide plus human gamma-globulin (Fig. 13 A). However, unlike glibenclamide which increased the spontaneous activity of the rat MPV on its own, lemakalim actually abolished the spontaneous activity of the vein at its higher concentration (5 μM) (Fig. 13 C). The interaction between lemakalim and human gamma-globulin appears to have produced a contracture of the vein resulting in a raised baseline tone of the vein. This raising of the baseline accounts for the increase in integrated response shown in Figure 10 and Table 3. Thus, lemakalim decreases the spontaneous activity of the portal vein in a concentration-dependent manner on its own and causes a transient increase in the frequency of contraction produced by human gamma-globulin as well as interacting with human gamma-globulin to increase the contracture or tone of the rat mesenteric portal vein.

3.5 In vitro effect of lemaklim on the action of noradrenaline and glibenclamide in the rat MPV

Figure 11 shows a lemakalim relaxation curve in the rat mesenteric portal vein. A concentration (0.5 μM) which caused roughly a 30% decrease in the spontaneous activity of the rat mesenteric portal vein significantly inhibited the stimulatory action of noradrenaline on the spontaneous contractility of the rat portal vein (bottom of fig. 11).
Figure 12 shows that lemakalim (0.5 µM) insurmountably blocked the simulatory action of glibenclamide on the spontaneous contractile activity of the rat mesenteric portal vein to a statistically significant level. This inhibition was not due to a vehicle effect as also is shown in figure 12.

3.6 *In vitro effects of prostaglandin inhibitors on the action of human gamma-globulin in the rat MPV*

Indomethacin (1x10⁻¹⁰ M and 1x10⁻⁶ M) (Fig. 14) and meclofenamic acid (1x10⁻¹⁰ M and 1x10⁻⁶ M) (Fig. 15) both significantly inhibited the stimulatory action of human gamma-globulin on the rat mesenteric portal vein in a concentration-dependent manner. Indomethacin was a more potent inhibitor of human gamma-globulin, decreasing the maximum integrated response of the rat MPV to gamma-globulin by 40% at its lower concentration (1x10⁻¹⁰ M) and 60% at its higher concentration (1x10⁻⁶ M) (Fig. 14). Meclofenamic acid, by comparison, only decreased the maximum integrated response of the portal vein to gamma-globulin by 15% at its lower (1x10⁻¹⁰ M) concentration and 52% at its higher concentration (1x10⁻⁶ M) (Fig. 15).

Phenylbutazone (1x10⁻⁶ M) (Fig. 16), aspirin (1x10⁻⁴ M) (Fig. 17), corticosterone (1x10⁻⁵ M) (Fig. 18), ibuprofen (1x10⁻⁶ M) (Fig. 19), and piroxicam (1x10⁻⁶ M) (Fig. 20) all tended to inhibit the stimulatory action of human gamma-globulin on the rat mesenteric portal vein in a concentration-dependent manner. However, only cortico-
sterone inhibited human gamma-globulin to a significant degree, decreasing the maximum integrated response of the portal vein to human gamma-globulin by 22% (Fig. 18). The order of potency with respect to inhibiting the stimulatory action of human gamma-globulin on the rat MPV for the prostaglandin inhibitors which were tested appears to be: indomethacin > meclofenamic acid > corticosterone > phenylbutazone > aspirin > ibuprofen > piroxicam (Figs. 14 to 20).

3.7 *Ex vivo effects of indomethacin on human-gamma globulin*

Figure 21 shows that a bolus infusion of indomethacin (10 mg/kg) *in vivo* significantly inhibits the action human gamma-globulin *in vitro*. Human gamma-globulin increased the spontaneous activity of the rat mesenteric portal veins *in vitro* exposed to a vehicle/volume control *in vivo* by 34% above baseline and only increased the activity of veins exposed to indomethacin *in vivo* by 18% (Fig. 21).
Figure 1 Effect of human gamma-globulin (hatched columns) on the rat mesenteric portal vein compared with control (Krebs, pH=7.6) (solid columns). The maximum integrated response of each preparation was set at 100% and all other responses were expressed as a percentage of this value. Each column represents the mean ± S.E.M.; n=6. * represents a statistically significant difference with respect to the control.
% Maximum Integrated Response

[Gamma-globulin], (mg/ml)

1.33  2.35  3.16  3.81  4.35  4.8

\*\*\*\*\*
Figure 2  Effect of human gamma-globulin (hatched columns) and histamine (cross-hatched columns) compared to volume/pH/time control (solid columns) on guinea-pig taenia-caecum. The numbers 1, 2, 3, 4, 5 and 6 (on the abscissa of figure 2) correspond to the following concentrations: human gamma-globulin as in figure 1 or histamine (M) 3x10^{-9}, 3x10^{-8}, 3x10^{-7}, 3x10^{-6}, 3x10^{-5}, and 3x10^{-4}. The maximum integrated response of each preparation was set at 100% and all other responses were expressed as a percentage of this value. Each column represents the mean ± S.E.M.; n=6. * represents a statistically significant difference with respect to the control.
Figure 3  Effect of human gamma-globulin (hatched columns) and noradrenaline (cross-hatched columns) on rat aortic rings without endothelium compared with volume/time control (solid columns). The numbers 1, 2, 3, 4, 5 and 6 (on the abscissa of figure 3) correspond to the following concentrations: human gamma-globulin as in figure 1 or noradrenaline (M) $1 \times 10^{-9}$, $1 \times 10^{-8}$, $1 \times 10^{-7}$, $1 \times 10^{-6}$, $1 \times 10^{-5}$, and $1 \times 10^{-4}$, respectively. Data are expressed as force of contraction in grams tension. Each column represents the mean ± S.E.M.; n=7. * represents a statistically significant difference with respect to the control.
Figure 4 Effect of human gamma-globulin (hatched columns) and noradrenaline (cross-hatched columns) on rat aortic rings with intact endothelium compared with volume/time control (solid columns). The numbers 1, 2, 3, 4, 5 and 6 (on the abscissa of figure 4) correspond to the following concentrations: human gamma-globulin as in figure 1 or noradrenaline as in figure 3. Data are expressed as force of contraction in grams tension. Each column represents the mean ± S.E.M.; n=7. * represents a statistically significant difference with respect to the control.
Force of Contraction (grams tension)

Concentration

1  2  3  4  5  6

-0.10  0.05  0.10  0.15  0.20  0.25  0.30  0.35  0.40  0.45  0.50  0.55  0.60  0.65
Figure 5  Effect of human gamma-globulin (hatched columns) and histamine (cross-hatched columns) compared with volume/time control (solid columns) in guinea-pig tracheal chains. The numbers 1, 2, 3, 4, 5 and 6 (on the abscissa of figure 5) correspond to the following concentrations: human gamma-globulin as in figure 1 or histamine as in figure 2. Data are expressed as force of contraction in grams tension. Each column represents the mean ± S.E.M.; n=7. * represents a statistically significant difference with respect to the control.
Figure 6 Noradrenaline concentration-response curve in the absence (circles) and following (squares) a bolus concentration of human gamma-globulin (4.35 mg/mL) on the rat mesenteric portal vein in vitro. Data are expressed as percent maximum integrated response with each point representing the mean ± S.E.M.; n=6.
Figure 7  Responses to human gamma-globulin alone (solid columns), human gamma-globulin in the presence of 5 μM glibenclamide (hatched columns), and responses to glibenclamide alone (5 μM) (cross-hatched columns) in the rat mesenteric portal vein. The three columns shown for the glibenclamide alone correspond to responses at 10, 14, and 18 minutes, respectively. Data are expressed as percent maximum integrated response with each point representing the mean ± S.E.M.; n=6. * represents a statistically significant difference with respect to the gamma-globulin control curve.
X-axis Legend

( ) = minutes of exposure to gibenclamide alone

[ ] = [Gamma-globulin], (mg/mL)
Figure 8  Concentration-response curve to human gamma-globulin in the presence (squares) and absence (circles) of 5 µM diazoxide. Data are expressed as percent maximum integrated response with each point representing the mean ± S.E.M.; n=6. * represents a statistically significant difference with respect to the gamma-globulin control curve.
Figure 9  Concentration-response curves to human gamma-globulin in the presence (squares) and absence (circles) of pinacidil. The concentration of pinacidil is 0.5 µM (top figure) and 5 µM (lower figure). Data are expressed as percent maximum integrated response with each point representing the mean ± S.E.M.; n=6. * represents a significant difference with respect to the gamma-globulin control curve.
Figure 10 Responses to human gamma-globulin in the rat mesenteric portal vein in vitro. Control concentration-response curves (circles). Responses (squares) in the presence of 0.5 µM (top figure) and 5 µM (lower figure) lemakalim. Data are expressed as percent maximum integrated response with each point representing the mean ± S.E.M.; n=6. * represents a significant difference with respect to the gamma-globulin control curve.
Figure 11  Lemakalim relaxation curve in the rat mesenteric portal vein in vitro (top figure). Noradrenaline concentration-response curves (lower figure) in the absence (circles) and presence (squares) of 0.5 µM lemakalim. Data are expressed as percent maximum integrated response with each point representing the mean ± S.E.M.; n=6. * represents a significant difference with respect to the noradrenaline control curve.
Figure 12  Concentration-response curves to glibenclamide alone (circles) and in the presence of 0.5 μM lemakalim (squares) in the rat mesenteric portal vein in vitro. Responses to vehicle control (triangles). Data are expressed as percent maximum integrated response with each point representing the mean ± S.E.M.; n=6. * represents a statistically significant difference with respect to the glibenclamide control curve.
Figure 13 Polygraph traces of the spontaneous activity and integrated activity of portal veins in the presence of gamma-globulin and glibenclamide (5 μM) (Panel A), or lemakalim (0.5 μM (Panel B) and 5 μM (Panel C)).
A

Integrated force

force

5 µM Glibenclamide

[Gamma-globulin], (mg/mL)

B

0.5 µM Lemakalim

[Gamma-globulin], (mg/mL)

C

5 µM Lemakalim

[Gamma-globulin], (mg/mL)
TABLE 1. Frequency of spontaneous contractions (number of spikes/minute) in the rat mesenteric portal vein.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Frequency (no. of spikes/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Krebs, pH 7.6)</td>
<td>6</td>
<td>3.65±0.3</td>
</tr>
<tr>
<td>Gamma-globulin control (4.35 mg/mL)</td>
<td>6</td>
<td>5.6±0.3 **</td>
</tr>
<tr>
<td>Noradrenaline control 1 x 10^-5 M</td>
<td>6</td>
<td>6.8±0.5 **</td>
</tr>
<tr>
<td>Gamma-globulin (4.35 mg/mL) + Noradrenaline</td>
<td>6</td>
<td>6.5±0.4</td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL) + Glibenclamide</td>
<td>6</td>
<td>13.8±2.3 *</td>
</tr>
<tr>
<td>(5 µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL) + Diazoxide (5 µM)</td>
<td>6</td>
<td>4.1±0.2 *</td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL) + Pinacidil (0.5 µM)</td>
<td>6</td>
<td>4.3±0.3 *</td>
</tr>
<tr>
<td>Gamma-globulin (4.35 mg/mL) + Pinacidil (5 µM)</td>
<td>6</td>
<td>2.1±1.1 *</td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL) + Lemakalim (0.5 µM)</td>
<td>6</td>
<td>Undetermined</td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL) + Lemakalim (5 µM)</td>
<td>6</td>
<td>Undetermined</td>
</tr>
</tbody>
</table>

NB: All values were calculated at the maximum response.
** = significantly different from control (Krebs, pH 7.6) value.
*  = significantly different from gamma-globulin control value.
TABLE 2. Force of spontaneous contractions (mean tension in grams) in the rat mesenteric portal vein.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Force (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Krebs, pH 7.6)</td>
<td>6</td>
<td>0.36±0.06</td>
</tr>
<tr>
<td>Gamma-globulin control (4.35 mg/mL)</td>
<td>6</td>
<td>0.63±0.08 **</td>
</tr>
<tr>
<td>Noradrenaline control 1 x 10^{-5} M</td>
<td>6</td>
<td>1.08±0.08 **</td>
</tr>
<tr>
<td>Gamma-globulin (4.35 mg/mL)+Noradrenaline</td>
<td>6</td>
<td>1.04±0.07</td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL)+Glibenclamide (5µM)</td>
<td>6</td>
<td>0.86±0.06 *</td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL)+Diazoxide (5µM)</td>
<td>6</td>
<td>0.60±0.06</td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL) + Pinacidil (0.5µM)</td>
<td>6</td>
<td>0.58±0.07</td>
</tr>
<tr>
<td>Gamma-globulin (4.35 mg/mL)+Pinacidil (5µM)</td>
<td>6</td>
<td>0.33±0.01 *</td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL)+Lemakalim (0.5µM)</td>
<td>6</td>
<td>0.51±0.03</td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL)+Lemakalim (5µM)</td>
<td>6</td>
<td>0.33±0.06 *</td>
</tr>
</tbody>
</table>

NB: All values were calculated at the maximum response.

** = significantly different from control (Krebs, pH 7.6) value.
*  = significantly different from gamma-globulin control value.
TABLE 3. Integrated response of spontaneous contractions (integrated response) in the rat mesenteric portal vein.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Integrated Response (tension in grams-min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Krebs, pH 7.6)</td>
<td>6</td>
<td>0.13±0.03</td>
</tr>
<tr>
<td>Gamma-globulin control (4.35 mg/mL)</td>
<td>6</td>
<td>0.39±0.04 **</td>
</tr>
<tr>
<td>Noradrenaline control 1 x 10⁻⁵ M</td>
<td>6</td>
<td>1.01±0.04 **</td>
</tr>
<tr>
<td>Gamma-globulin (4.35 mg/mL)+Noradrenaline</td>
<td>6</td>
<td>0.86±0.06</td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL)+Glibenclamide (5 µM)</td>
<td>6</td>
<td>0.74±0.04 *</td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL)+Diazoxide (5 µM)</td>
<td>6</td>
<td>0.22±0.04 *</td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL)+Pinacidil (0.5 µM)</td>
<td>6</td>
<td>0.26±0.02 *</td>
</tr>
<tr>
<td>Gamma-globulin (4.35 mg/mL)+Pinacidil (5 µM)</td>
<td>6</td>
<td>0.11±0.01 *</td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL)+Lemakalim (0.5 µM)</td>
<td>6</td>
<td>0.43±0.04</td>
</tr>
<tr>
<td>Gamma-globulin (2.35 mg/mL)+Lemakalim (5 µM)</td>
<td>6</td>
<td>0.54±0.03 *</td>
</tr>
</tbody>
</table>

NB: All values were calculated at the maximum response.

** = significantly different from control (Krebs, pH 7.6) value.
* = significantly different from gamma-globulin control value.
Figure 14  Concentration-response curves to human gamma-globulin in the presence (hatched columns) and absence (solid columns) of indomethacin. The concentration of indomethacin is $1 \times 10^{-10}$ M (top figure) and $1 \times 10^{-6}$ M (lower figure). Data are expressed as percent maximum integrated response with each column representing the mean ± S.E.M.; n=6. * represents a significant difference with respect to the gamma-globulin control curve.
Figure 15  Concentration-response curves to human gamma-globulin in the presence (hatched columns) and absence (solid columns) of meclofenamic acid. The concentration of meclofenamic acid is $1 \times 10^{-10}$ M (top figure) and $1 \times 10^{-6}$ M (lower figure). Data are expressed as percent maximum integrated response with each column representing the mean ± S.E.M.; n=6. * represents a significant difference with respect to the gamma-globulin control curve.
Figure 16 Concentration-response curve to human gamma-globulin in the presence (hatched columns) and absence (solid columns) of $1 \times 10^{-6}$ M phenylbutazone. Data are expressed as percent maximum integrated response with each column representing the mean $\pm$ S.E.M.; $n=6$. * represents a statistically significant difference with respect to the gamma-globulin control curve.
Figure 17 Concentration-response curve to human gamma-globulin in the presence (hatched columns) and absence (solid columns) of $1 \times 10^{-4}$ M aspirin. Data are expressed as percent maximum integrated response with each column representing the mean ± S.E.M.; n=6. * represents a statistically significant difference with respect to the gamma-globulin control curve.
Figure 18 Concentration-response curve to human gamma-globulin in the presence (hatched columns) and absence (solid columns) of $1 \times 10^{-5}$ M corticosterone. Data are expressed as percent maximum integrated response with each column representing the mean $\pm$ S.E.M.; $n=6$. * represents a statistically significant difference with respect to the gamma-globulin control curve.
Figure 19  Concentration-response curve to human gamma-globulin in the presence (hatched columns) and absence (solid columns) of 1x10^{-6} M ibuprofen. Data are expressed as percent maximum integrated response with each column representing the mean ± S.E.M.; n=6. * represents a statistically significant difference with respect to the gamma-globulin control curve.
% Maximum Integrated Response

[Gamma-globulin], (mg/ml)

1.33 2.35 3.16 3.81 4.35 4.8
Figure 20  Concentration-response curve to human gamma-globulin in the presence (hatched columns) and absence (solid columns) of 1x10^{-6} M piroxicam. Data are expressed as percent maximum integrated response with each column representing the mean ± S.E.M.; n=6. * represents a statistically significant difference with respect to the gamma-globulin control curve.
Figure 21 Ex vivo experiment. Concentration-response curve to human gamma-globulin in vitro on rat mesenteric portal veins exposed to 10 mg/kg of indomethacin in vivo (hatched columns) or the equivalent volume vehicle control in vivo (solid columns) one hour previously. Data are expressed as percentage change from baseline with each column representing the mean ± S.E.M.; n=6. * represents a statistically significant difference with respect to the gamma-globulin control curve.
4 DISCUSSION

4.1 Stimulant effect of human gamma-globulin on smooth muscle preparations

Calcium and/or potassium channels may be related to spontaneous contractile activity. Calcium channels can be directly activated to increase calcium conductance and potassium channels can be inactivated to reduce conductance resulting in continued depolarization (Triggle et al., 1992). Both these changes in permeability would lead to increased spontaneous contractility. The fact that certain smooth muscles lack spontaneous activity (i.e. are quiescent) may be due to the presence of outwardly rectifying potassium channels.

In the present study, human gamma-globulin was shown to have a stimulant effect on the spontaneous contractile activity of the rat mesenteric portal vein, exerting its maximum effect at a concentration of 4.35 mg/ml, which is consistent with a previous report from our laboratory (Pillai and Sutter, 1990). Although the human gamma-globulin used in these and previous experiments (Pillai and Sutter, 1990) is not a homogenous substance, it has an electrophoretic purity of approximately 99% and our laboratory has previously identified immunoglobulin IgG as the active substance in this source of human gamma-globulin, which causes a concentration-dependent increase in spontaneous activity of the rat mesenteric portal vein (Pillai and Sutter, 1990). In addition, we used three
different batches of human gamma-globulin during the course of this thesis, all of which were stimulatory. It was also previously observed in our laboratory (Pillai and Sutter, 1990) that alpha- and beta-globulins are both inhibitory. It is, therefore, unlikely that the stimulant effect of human gamma-globulin is due to a contaminant or due to globulin per se.

Human gamma-globulin caused a concentration-dependent increase in the spontaneous activity (both amplitude and frequency) of both the rat mesenteric portal vein and the guinea-pig taenia-caeci (both being spontaneously-active muscles). Human gamma-globulin did not significantly affect the contractile activity of either the rat aorta (with or without endothelium) or the guinea-pig trachea (both being quiescent muscles). These findings are consistent with the view that immunoglobulins act by modifying membrane electrical activity and suggest that they may modulate ion channels associated with spontaneous contractions.

4.2 Effects of diazoxide, pinacidil, lemakalim (BRL 38227) and glibenclamide on the actions of human gamma-globulin in the rat mesenteric portal vein

In this study, we examined the effect of human gamma-globulin on responses to noradrenaline and found no significant effect. This suggests that human gamma-globulin and noradrenaline act at different sites since there was neither inhibition nor potentiation of the effects of noradrenaline on the rat mesenteric portal vein by human
gamma-globulin. It has been shown in isolated cells of the rat and rabbit portal veins that depolarization in response to noradrenaline is mediated by an increase in chloride conductance which is dependent upon both the calcium release from intracellular stores and the increase of the voltage-dependent calcium current (Pacaud et al., 1989; Large, 1989). Thus, these observations may be taken to imply that human gamma-globulin does not exert its stimulatory effect on the spontaneous activity of the rat mesenteric portal vein by modulation of a chloride channel, since noradrenaline acts on a chloride channel.

The main aim of this study was to determine whether or not human gamma-globulin stimulates the spontaneous contractility of the rat mesenteric portal vein by inactivating potassium channels. In order to investigate this possibility, we constructed concentration-response curves to human gamma-globulin in the presence of glibenclamide, a potassium channel blocker, and the potassium channel activators diazoxide, pinacidil, and lemakalim (BRL 38227). Glibenclamide is a hypoglycemic sulfonylurea which is used clinically in the treatment of diabetes mellitus (Loubatieres, 1977; Jackson and Bressler, 1981). Sulfonylureas act primarily by blocking ATP-sensitive potassium channels in the pancreatic \( \beta \)-cells leading to \( \beta \)-cell membrane depolarization (Ferrer et al., 1984; Schmid-Antomarchi et al., 1987; Fosset et al., 1988). Sulfonylureas also block ATP-sensitive potassium channels in
cardiac ventricular myocytes (Sanguinetti et al., 1988; Escande et al., 1989) and some peripheral arteries (Standen et al., 1989).

In the present study, we found that glibenclamide (5 \(\mu\)M) had a biphasic effect on the spontaneous contractile activity of the rat mesenteric portal vein - first increasing and then decreasing the spontaneous activity (frequency, force, and integrated response). This may explain the discrepant effects of glibenclamide on the spontaneous activity of the rat mesenteric portal vein which have been reported in the literature. Glibenclamide has been variously reported to slightly increase the spontaneous activity of the rat portal vein (Winquist et al., 1989; Longmore et al., 1990), decrease the spontaneous activity of the rat mesenteric portal vein (Quast and Cook, 1989), or have no effect on the spontaneous activity of the rat mesenteric portal vein (Buckingham et al., 1989; Winquist et al., 1989; Schwietert et al., 1992). We found that glibenclamide (5 \(\mu\)M) significantly increased the stimulant action of human gamma-globulin on the spontaneous activity (frequency, force, and integrated response) of the rat mesenteric portal vein. This observation supports our hypothesis that human gamma-globulin exerts its stimulatory effects by decreasing potassium conductance. In addition, it was observed that human gamma-globulin when added to the bath with glibenclamide increased the tone or contracture of the portal vein.
In addition to glibenclamide, we examined the effects of three potassium channel activators (diazoxide, pinacidil, and lemakalim) on the action of human gamma-globulin in the rat mesenteric portal vein. Potassium channel activation has recently been recognized as the cellular basis involved in the relaxation of smooth muscle by diazoxide, pinacidil, and lemakalim (Bray et al., 1987; Black et al., 1990; Triggle et al., 1992). These three compounds are from a structurally diverse group of antihypertensive drugs known as potassium channel openers (PCOs) (for reviews see Edwards and Weston, 1990; Quast, 1992). The relaxation of smooth muscle mediated by this group of drugs has been shown to be blocked by glibenclamide (Escande et al., 1989; Standen et al., 1989; Winquist et al., 1989; Eltze, 1989). Diazoxide, a potassium channel opener which is used clinically, has been shown to open ATP-sensitive potassium channels in pancreatic β-cells (Zunkler et al., 1988; Dunne et al., 1989) and its smooth muscle relaxing action is blocked by glibenclamide (Newgreen et al., 1989). These reports have lead to the tentative conclusion that potassium channel openers in vascular smooth muscle activate a potassium channel similar to the ATP-sensitive potassium channel described for the pancreatic β-cell membrane. In spite of the circumstantial evidence linking the action of potassium channel openers to an ATP-sensitive potassium channel, little if any direct evidence exists.
In the rat mesenteric portal vein, pinacil has been shown to produce a concentration-dependent relaxant effect that is antagonised by procaine and tetraethylammonium, but not 3,4-diamino-pyridine (Southerton et al., 1988). Pinacidil has been shown to activate an ATP-sensitive potassium channel in rabbit portal vein cells (Kajioka et al., 1991). However, the antagonism by glibenclamide of the relaxant responses to pinacidil has been shown to be non-competitive, which suggests that it acts at sites different from those acted upon by pinacidil (Masuzawa et al., 1990). In the rat mesenteric portal vein, pinacidil (0.3-100 μM) has been shown to inhibit the spontaneous contractile activity and responses to noradrenaline (0.001-100 μM) (Weston et al., 1988).

In this study, we found that both diazoxide and pinacidil insurmountably blocked the stimulant action of human gamma-globulin on the spontaneous activity of the rat mesenteric portal vein to a significant extent in a concentration-dependent manner. This observation is also consistent with our theory that human gamma-globulin exerts its stimulatory effect by blocking potassium channels. However, the fact that the block was non-competitive suggests that human gamma-globulin is acting at a site different from that of diazoxide and pinacidil.

Lemakalim (BRL 38227), a novel potassium channel opener, is the active form (L-enantiomer) of cromakalim (BRL 34915) (Buckingham et al., 1986; Hof et al., 1988; Post et
In the present study, we have shown that lemakalim antagonizes the stimulatory actions of both noradrenaline and glibenclamide in the rat mesenteric portal vein, which is consistent with the findings of Noack et al. (1992). In addition, we found that lemakalim abolishes the spontaneous activity of the rat mesenteric portal vein in a concentration-dependent manner. However, we also observed that lemakalim when added to the bath with human gamma-globulin produced an effect similar to that observed when glibenclamide was added to the bath with human gamma-globulin. In both instances, an increase in the contracture or baseline tone of the portal vein producing a significant initial and transient increase in the frequency of contractions produced by human gamma-globulin followed by a cessation of spiking frequency was observed. Thus, lemakalim appears to increase the stimulatory action of human gamma-globulin on the rat mesenteric portal vein as measured by integrated response. This observation is in direct conflict with our hypothesis which predicted that all potassium channel openers would inhibit the stimulatory action of human gamma-globulin on the rat mesenteric portal vein. However, this is an important observation because it emphasizes the fact that spontaneous activity and contracture are two distinct entities.

There are at least 13 major types of potassium channels which are currently recognized (Weston et al., 1990), so that interaction with potassium channels in the rat
mesenteric portal vein other than the ATP-sensitive channel must be considered not only for human gamma-globulin, but also for glibenclamide, diazoxide, and pinacidil. Recently it was reported that glibenclamide can block calcium-dependent potassium channels (Gelband et al., 1990). Additionally, it should be noted that relatively high (>1 µM) concentrations of glibenclamide are required to inhibit the relaxant effects mediated by potassium channel openers in smooth muscle compared with pancreatic cells (Schmid-Antomarchi et al., 1987; Triggle et al., 1992). One explanation for the heterogeneity of action of potassium channel openers is the existence of subtypes of ATP-sensitive potassium channels in smooth muscle (Wickenden et al., 1991). One study on the relaxant action of potassium channel openers, including pinacidil, on rat oesophageal smooth muscle has demonstrated that potassium channel openers are sensitive to inhibition by nifedipine-like drugs (Akbarali et al., 1988). Other studies have reported that some potassium channel openers mediate smooth muscle relaxation by interfering with intracellular calcium release (Meisheri et al., 1991; Xiong et al., 1991). Triggle et al. (1992) have suggested that functional ATP-sensitive potassium channels in smooth muscle vary in a tissue- and probably species-dependent manner. Support for this view comes from recent studies showing that not all vascular smooth muscle preparations respond to potassium channel openers (Wickenden et al., 1991). However, an earlier study
reported that the potassium channel openers show little selectivity for different types of smooth muscle (Hamilton and Weston, 1989).

Recently, E-4031, which is a sotalol derivative and a selective blocker of the delayed rectifier current \( (I_k) \) in cardiac tissue (Colatsky and Follmer, 1989; Wettwer et al., 1991), was shown to significantly increase the contractile activity of the rat portal vein (Schwietert et al., 1992). This suggests voltage-sensitive potassium channels have an important functional role in the repolarization of pacemaker cells in the rat portal vein. Many different potassium channels have been described in the rat portal vein (Hu et al., 1990; Kajioka et al., 1990; Okabe et al., 1989) with the voltage and calcium dependent maxi-K\(^+\) channel being the most prominent in the whole-cell configuration (Hu et al., 1990).

4.3 **Effect of prostaglandin inhibitors on the action of human gamma-globulin in the rat mesenteric portal vein**

It has been suggested that prostaglandins can alter calcium availability for muscle contraction (Northover, 1968) and that they may play a role in the spontaneous active tone of blood vessels (Daniel and Sarna, 1978). Prostaglandins are found in tissues throughout the body but their physiological roles are not fully understood (Neal, 1987). Enero (1979) reported that meclofenamate (10 \( \mu \)M) depressed the spontaneous contraction of the rat portal vein by approximately 50% without modifying the response to
noradrenaline. Meclofenamate inhibits both phosphodiesterase and prostaglandin activities (Flower, 1974). Ouabain has been shown to inhibit the contractile effects of prostaglandin \( F_{1\alpha} \) but not that of prostaglandin \( F_{2\alpha} \) (Kadar and Sunahara, 1969), which suggests that prostaglandins can constrict blood vessels by more than one mechanism.

In order to determine if prostaglandins play a role in the stimulatory effect of human gamma-globulin on the spontaneous activity of the rat mesenteric portal vein, we constructed concentration-response curves to human gamma-globulin in the presence of various NSAIDs as well as corticosterone. NSAIDs form a chemically diverse group of compounds, all of which have the ability to inhibit cyclooxygenase resulting in the inhibition of prostaglandin synthesis. Corticosterone inhibits phospholipase \( A_2 \) and consequently inhibits the formation of leukotrienes as well as prostaglandins. We tested NSAIDs from each of the different chemical groups: propionic acids (ibuprofen), acetic acids (indomethacin), fenamates (meclofenamic acid), oxicams (piroxicam), and pyrazolones (phenylbutazone) (Neal, 1987). We found that all the NSAIDs tested, as well as corticosterone, had an inhibitory effect on the stimulatory action of human gamma-globulin on the spontaneous activity of the rat mesenteric portal vein. However, only indomethacin, meclofenamic acid, and corticosterone inhibited the stimulatory action of human gamma-globulin to a significant extent. Both indomethacin and meclofenamic
acid were observed to inhibit the spontaneous activity of the portal vein at their higher concentration ($1 \times 10^{-6}$ M). Indomethacin and meclofenamic acid were both more potent inhibitors of the stimulatory action of human gamma-globulin than corticosterone, which suggests that prostaglandins play a more important role in the stimulatory action of human gamma-globulin than do leukotrienes. Additionally, we did an ex vivo experiment with a relatively high dose of indomethacin (10 mg/kg) and found that it caused a decrease in spontaneous activity of the rat mesenteric portal vein as well as inhibiting the response of the portal vein to human gamma-globulin by 16%.

5 SUMMARY

This thesis was comprised of three separate studies whose aim was to examine the nature of human gamma-globulin stimulation of spontaneous activity (frequency, force, and integrated response) in the rat mesenteric portal vein. The first study was designed to determine whether or not human gamma-globulin exerts its stimulatory effect only on smooth muscles with spontaneous activity. The second study was designed to examine whether human gamma-globulin stimulates spontaneous contractility in the rat mesenteric portal vein by inactivating potassium channels. The third study was designed to investigate whether or not prostaglandins play a role in the stimulatory action of human gamma-globulin on the rat mesenteric portal vein.
Results from the first study show that human gamma-globulin exerts its stimulatory effect only on spontaneously active smooth muscles but not on quiescent muscles. The results from the second study show that human gamma-globulin has no effect on the action of noradrenaline on the rat mesenteric portal vein, that glibenclamide has a biphasic effect on the spontaneous activity of the rat mesenteric portal vein, that glibenclamide potentiates the stimulant action of human gamma-globulin on the rat mesenteric portal vein, that the potassium channel openers diazoxide and pinacidil both insurmountably inhibit the action of human gamma-globulin on the rat mesenteric portal vein, that lemakalim antagonizes the actions of both noradrenaline and glibenclamide on the rat mesenteric portal vein, that lemakalim abolishes the spontaneous activity of the rat mesenteric portal vein in a concentration-dependent manner, and that lemakalim potentiates the stimulatory action of human gamma-globulin on the integrated force of contraction in the rat mesenteric portal vein in a manner similar to glibenclamide by increasing the tone of the vein.

Controversy surrounding the exact site of action of glibenclamide, pinacidil, diazoxide, and lemakalim in the rat mesenteric portal vein make it difficult to ascertain where human gamma-globulin is acting. From this study, it appears that human gamma-globulin acts at a site different from noradrenaline, diazoxide and pinacidil, but possibly at a site affected by the actions of glibenclamide and
lemakalim. Findings from this study may be taken to suggest that human gamma-globulin, which is a protein, may act by directly modulating a potassium channel such as the maxi-K⁺ channel. Further studies, at the cellular level, such as whole cell electrophysiological studies on freshly dispersed cells are needed to clarify these observations.

The third study in this thesis showed that prostaglandin inhibitors block the stimulatory action of human gamma-globulin on the spontaneous activity of the rat mesenteric portal vein in vivo as well as ex vivo.

In summary, the findings of this thesis are consistent with the view that immunoglobulins act by modifying membrane electrical activity and suggest that they may modulate ion channels associated with spontaneous contractions. It appears that human gamma-globulin exerts its stimulatory action on the spontaneous activity of smooth muscle by decreasing potassium conductance. It also appears that prostaglandins play a role in the stimulatory action of human gamma-globulin on the rat mesenteric portal vein.
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