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BRADYKININ INDUCED CONTRACTION OF HUMAN GALLBLADDER MUSCLE *IN VITRO.*

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Abstract

Some patients with gallstones have impaired gallbladder emptying, although it is not known whether this is primary or secondary to inflammation. Reduced contractility in human gallbladder muscle with severe inflammation has been demonstrated *in vitro*, one of the earliest events in this process is release of such vasodilators as histamine, prostacyclin PGI_2 and bradykinin (BK), all of which serve to increase the blood supply to the affected area. BK is an inflammatory mediator with widespread regulatory functions throughout the GI tract. The effects of BK on a variety of tissue had been well documented. The initial work for this thesis looked at the effects of bradykinin on human gallbladder contractility. The aim was to determine if BK had an effect on human gallbladder tissue and if so, what BK receptors were involved and to investigate if other mechanisms had an influence on this effect.

Contractility tests were used to examine the BK receptor types, selective antagonists of BK1 and BK2 were used. Stone bearing gallbladders were obtained from patients undergoing cholecystectomy for gallstone disease and healthy gallbladders were removed from patients undergoing resections for pancreatic or hepatic malignancy. Only macroscopically normal tissue was used. A full thickness segment from the anterior aspect of the body of the gallbladder was removed and placed in cold Krebs solution. Muscle strips were cut from the segment and suspended in Krebs solution in an organ bath at 37^0C, gassed with 5% CO_2 in oxygen. The strips were connected to an isotonic transducer under a 1g pre-load. After 2 hours equilibration, a concentration-response curve to BK was obtained (0.1μM-100μM), and washed out until a stable baseline had returned.

It was determined that BK contracted human gallbladder smooth muscle *in vitro* and that both BK1 and BK2 receptors were involved in this response. It was argued that a difference in receptor population exists between normal and stone-bearing tissue. It was also suggested that another class of BK receptor maybe present. There is also evidence to suggest that a third BK receptor or another independent mechanism may mediate this response.

Having established the initial results, the interaction of the BK response with various other substances, mediators and mechanisms known to have an effect on gallbladder motility had to be explored. BK appears to have a direct effect on human gallbladder smooth muscle. The BK-mediated contraction of human gallbladder depends on the release of intracellular calcium sources. The contraction is mediated by release of a cyclo-oxygenase pathway derived product.

BK is believed to play an important role in inflammation and inflammatory pain. Inflammation is a pre-cursor to gallstone formation. The *in vitro* studies reported in this thesis will give an insight into the role played by BK in the process of gallbladder motility. Studies have shown the powerful analgesic effect of non-steroidal anti-inflammatory drugs when used in biliary disease, suggesting an important role for prostaglandin in the symptoms related to gallstones. Further elucidation of the role of BK in gallbladder contraction may help improve therapy of gallstone disease – the work described here, attempts to do so.

TABLE OF CONTENTS

Abstract		
Contents		**iii**
Acknowledgements		**vi**
Dedication		**vii**
Publications and Presentations		**viii**
Declaration		**x**
1.	**INTRODUCTION**	**1**
	• **The Gallbladder**	
	• **Gallstone Disease**	
	• **Bradykinin**	
	• **Cholecystokinin**	
2.	**REVIEW OF LITERATURE**	**9**
2.1	**Gallbladder**	**9**
2.1.1	**Gallbladder Motility**	**9**
2.1.2	**Gallstone Formation**	**11**
2.2	**Kinins**	**12**
2.2.1	**Overview**	**12**
2.2.2	**Historical Background**	**15**
2.2.3	**Actions**	**17**
	2.2.3.1 Kinins	**18**
	2.2.3.2 Bradykinin	**20**
2.3	**Bradykinin**	**23**
2.3.1	**Background**	**23**
2.3.2	**Bradykinin Formation**	**25**
2.3.3	**Receptor Types**	**28**
2.3.4	**Functions/Actions**	**33**
2.3.5	**Mechanisims of Action**	**35**
2.4	**AIMS**	**38**

3.	MATERIALS & METHODS	39
3.1	Introduction	39
3.2	Disclaimer and consent	39
3.3	*In vitro* investigation	40
3.3.1	Tissue Type	40
3.3.2	Krebs Bicarbonate solution	41
3.3.3	Preparation of BK and Other peptides	42
3.3.4	Experimental Protocol	42
3.3.5	The organ bath and transducer	44
3.3.6	Viability Testing	45
3.3.7	Generation of a Concentration Response Curve	46
3.3.8	The *in vitro* study	47
3.3.9	Statistical Analysis	49
3.4	Measurement of Cholecystokinin	50
3.4.1	Patients	50
3.4.2	Study Protocol	50
3.4.3	CCK assay	51
3.4.4	Statistical analysis	52
3.5	*In vivo* V *In vitro* study	53
4.	RESULTS	54
4.1	Bradykinin and bradykinin receptor activity	55
4.1.1	Bradykinin dose response curves	55
4.1.2	Bradykinin receptor antagonists	59
4.1.3	Responses in normal and in the presence of gallstones	59
4.1.4	BK1 and BK2 receptors combined	62
4.1.5	Bradykinin 1 receptor agonist	64
4.1.6	Maximum BK response	65
4.1.7	Viability testing	69
4.2	Independent mechanisms of BK contraction	70
4.2.1	Cholecystokinin	70
4.2.1.1	CCK dose response curve and BK receptor interaction	72
4.2.1.2	CCK receptor	75
4.2.1.3	Possible BK and CCK receptor interaction	76
4.2.2	Various other possible receptors, channel mediators	77

4.3 ***In vivo* V *In vitro* argument** **86**

5. **DISCUSSION** **90**

6. **REFERENCES** **100**

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Dedication

I wish to dedicate this thesis to my parents. Their support was always there in every way, not just in this project and my education but all aspects of my life. A few words can never express my debt of gratitude.

Love and thanks. Hugh.

Publications and Presentations

The publications and presentations listed below are all related to the work described in this thesis – induced contraction of human gallbladder muscle. The titles tend to be similar as the work was ongoing and each abstract/presentation describes a separate piece of the work with the ongoing research.

Abstract Publications

Brennan H.J., Pearce N.W. and Johnson C.D. GUT 1998, 42, (supp 1) AII,T41.

Brennan H.J., Pearce N.W. and Johnson C.D. GUT 1998, 42, (supp 11)A33,TW129.

Brennan H.J., Pearce N.W. and Johnson C.D. J. of Physiol. 1998, 509, 16P.

Brennan H.J., Pearce N.W., Cleverly J. and Johnson C.D. J. of Physiol. 1998, 509, 17P.

Brennan H.J., Pearce N.W. and Johnson C.D. Hepato-Gastroenterology 1998, 45, (supp 11).

Pearce N.W., Brennan H.J and Johnson C.D. Br. J. of Surg 1998, 85,11,1589.

Pearce N.W., Brennan H.J, Cleverely J. and Johnson C.D. Br. J. of Surg 1998, 85,11,1590.

Brennan H.J., Pearce N.W. and Johnson C.D. J. of Physiol. 1998, 513, 94P.

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Presentations

British Society of Gastroenterology, Harrogate, March 1998.

The Physiology Society, Liverpool, April 1998.

South West & Wessex Surgeons, Taunton, May 1998.

IHBPA, Madrid, May 1998.

Surgical Research Society, Dublin, July 1998.

The Physiology Society, Southampton, September 1998.

American Gastroenterological Association, Orlando, Florida, 1999.

The Physiology Society, Newcastle, July 1999.

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Surgical Research Society, London, December 1999.

1. Introduction

When life appeared on this planet, about three billion years ago, injury and death came with it. The organisms developed structures and systems to assure their nutrition and reproduction as well as defence against noxious stimuli. In evolving animals, the reaction to injury became complex and involved various cellular, immunologic and biochemical mechanisms (Ryan and Majno 1977).

The Gallbladder

***gallbladder**,n. a bag-like reservoir (of 50cm^3 capacity in man) that lies at the edge of the liver closest to the gut and whose function is to store bile produced by the liver. The contents of the gallbladder are squirted into the gut lumen under the influence of the hormone cholecystokinin-pancreozymin.* (Hale and Margham 1988).

The most potent stimulus for emptying the gallbladder is the hormone cholecystokinin (CCK). Both gallbladder and CCK are discussed in detail in the review of literature section. Emptying of the gallbladder contents into duodenum begins several minutes after the start of a meal. CCK is released by the duodenal mucosa primarily in response to the presence of fats and their digestion products and essential amino acids in the duodenum. CCK reaches the gallbladder via the circulation and it causes strong contractions of the gallbladder and relaxation of the sphincter of Oddi. Between meals the tone of the sphincter of Oddi, which guards the entrance of the common bile duct into the duodenum, is high. Thus bile flow is diverted into the gallbladder.

The gallbladder is a small organ and has a capacity of 15-60ml (average 35ml) in humans. Many times this volume of bile may be secreted by the liver between meals. The gallbladder concentrates the bile by absorbing Na^+, Cl^-, HCO^-_3 and water from the bile so that the bile acids can be concentrated from 5 to 20 times in the gallbladder. K^+ is concentrated in the bile when the water is absorbed, and then K^+ is absorbed by simple diffusion. The active transport of Na^+ is the primary active process in the concentrating action of the gallbladder; Cl^- and HCO^-_3 are absorbed to preserve electro-neutrality.

Gallstone Disease

It has long been recognised that an inflammatory response involves a complex set of interactions. The pathogenesis of gallstone formation is complex. Some patients have impaired gallbladder emptying, although it is not known whether this is primary or secondary to inflammation. Reduced contractility in human gallbladder muscle with severe inflammation has been demonstrated *in vitro* (McKirdy et al. 1994). In gallstone disease the initial cellular damage that brings about the process of inflammation in the gallbladder is the result of chemical irritation of the mucosal lining. The disruption of the normally protective glycoprotein mucus layer exposes the epithelium to the potent and direct detergent action of bile salts. This constitutes the cellular insult necessary to initiate the inflammatory process. One of the earliest events in this process is release, of such vasodilators as histamine, prostacyclin (PGI_2) and bradykinin (BK), all of which serve to increase the blood supply to the affected area. These agents relax vascular smooth muscle and histamine and prostacyclins can

reduce contractility of the gallbladder (Kotall et al. 1984). The actions of BK on human gallbladders were unknown before experiments in our laboratory, which showed, BK-induced contraction of gallbladder (Johnson et al.1997). This study aims to investigate the mechanisms involved in BK induced contraction of human gallbladder muscle.

Bradykinin

Bradykinin (BK) is the most well studied of a group of peptides known as kinins. It is a nonapeptide generated in plasma by the action of plasma and glandular kallikreins on both high and low molecular weight kininogens (Regoli and Barabe 1980). The effects produced by BK are due to the activation of specific receptors. The existence of two BK receptors (BK1 and BK2) has been well documented and both have been intensively studied in recent years. They are both of the Rhodopsin type consisting of seven transmembrane hydrophobic helical segments by extracellular and intracellular loops.

BK exerts its effect by activating these two G-protein coupled receptors. Stimulation of BK1 and BK2 produce various different actions. These actions can vary dependent on tissue type and/or species. Yoshida et al. (1997) concluded that bradykinin exerts versatile actions in diverse tissues and cells through specific BK1 or BK2 receptors. Molecular biological studies revealed the primary structure of each BK receptor from various species. Feres et al. (1992) demonstrated two populations of bradykinin receptors in rat duodenum: a BK2 subtype

responsible for the biphasic response of the non-stretched duodenum, and a BK1 subtype responsible for the contractile effect on the stretched tissue.

BK can act both directly and indirectly on smooth muscle. In many organs, BK acts indirectly on the smooth muscle by promoting the release of other mediators, such as endothelium-derived relaxing factors in arteries (Sung et al. 1988), neurotransmitters from cholinergic (Yau et al. 1986), adrenergic (Rifo et al. 1987) or nonadrenergic-noncholinergic (NANC) motor nerves or from sensory nerves (Ueda et al. 1984), prostaglandins and leukotrienes from the targeting of neighbouring cell (Nasjletti and Malik 1979) and histamine or other autacoids from mast cells (Regoli et al. 1990). BK is also believed to act directly on the smooth muscle e.g. in the rabbit jugular vein and the rabbit aorta devoid of endothelium (Regoli et al. 1990, Farmer et al. 1989ii). In many tissues BK exerts several effects simultaneously producing a complex response that is the sum of several of the above actions (Regoli et al. 1990).

The BK1 receptors exhibit higher affinity for kinin metabolites e.g. des-Arg^9-BK and are selectively and competitively antagonised by the BK1 receptor antagonist, des-Arg^9-Leu^8-BK. However most of the physiological and pathological actions of BK are mediated by activation of BK2 receptors which seem to be widely distributed throughout the peripheral and central nervous system. BK2 receptors exhibit a high affinity for BK but a low affinity for the BK1 agonist, des-Arg^9-BK (Bathon and Proud 1991). The most potent BK2 receptor antagonist, HOE-140, has been already tested in models involving the action of endogenous kinins (Legat et al. 1994).

BK1 receptors are defined as those that respond to the following agonists with the rank order of agonist potency of:

[des-Arg^9-BK] > [Tyr(Me)^8-BK] > BK

along with an appreciable affinity of one of the BK1 receptor antagonists e.g. des-Arg^9-Leu^8-BK (pA_2 or pKb = 6.3-7.5).

The BK2 receptors respond to agonists with the rank order of potency:

[Tyr(Me)^8-BK] > BK > [des-Arg^9-BK]

having the affinity for a BK2 receptor antagonist e.g. HOE-140 (pA_2 or pKb = 8.5-10.5). Genes encoding the BK1 receptor have been cloned e.g. in the rabbit (MacNeil et al. 1995), human (Menke et al. 1994) and mouse (McIntyre et al. 1993). The cloned receptor has a predicted sequence of 353 amino acids and shows an overall sequence homology with the BK2 receptor of just 36% (Menke et al. 1994). A human BK2 receptor was cloned from a lung fibroblast cell line (Hess et al.1994). The cDNA clone encodes a 364 amino acid sequence protein that had the characteristics of a seven transmembrane domain G-protein receptor. The amino acid sequence of the human BK2 receptor is 81% identical to the BK2 receptor of rat smooth muscle (Abdalla et al. 1996).

Characteristics of BK1 and BK2 receptors.

	BK1	BK2
Receptor class	7-Transmembrane	7-Transmembrane
Amino Acids	353	364
Homology BK1/BK2	36%	36%
Pattern of expression	inducible	constitutive
Desensititization	no	yes
Internalization	no	yes

Cholecystokinin (CCK)

CCK is one of three classical gut hormones and was named for its ability to cause gallbladder contraction (Ivy and Oldberg 1928). The factors that control the release of CCK are complex and not fully understood. CCK may be involved in the pathophysiology of several gastrointestinal disease processes. CCK acts on the gallbladder smooth muscle cells directly (Schjoldager et al 1989) causing calcium dependent contraction (Grace et al 1990). Myers et al (1992) conclude that BK and not CCK stimulated exaggerated prostanoid release from the inflamed rabbit gallbladder. Any relationship between BK action and the CCK mode of action should be explored.

As far back as 1979 Paegelow et al reported that BK stimulates the influx of calcium ions into muscle cells of the ileum, it is ineffective if no extracellular calcium ions are available.

Masters et al. 1999 confirmed that internal stores contribute a significant amount of calcium in human muscle of the urinary bladder wall. Wassdal et al. (1999) concluded that when BK was applied to single smooth muscle cells from rat duodenum, cytosolic calcium was increased as a result of the emptying of intracellular calcium stores and by contribution from extracellular calcium. Hertog et al. (1988) showed multiple actions of BK mediated via BK2 receptors on calcium mobilisation associated with activation of potassium channels and on calcium release from intracellular stores and on receptor-activated sodium channels. This last example shows how complex the BK mediated response is – calcium, potassium and sodium are all involved in the BK2 mediated response. Sodium and potassium are two of the most important electrolytes. The role of these electrolytes in the human body is manifold. There are almost no metabolic processes that are not dependent on or affected by electrolytes. Among their functions is the regulation of the proper function of muscles, therefore they are important in gallbladder motor function. Shibuya et al (1999) suggested that the prostaglandin E2 causes calcium release from ryanodine sensitive stores through a mechanism independent of IP3 and cAMP, therefore a link between BK, calcium and prostglandins may exist and a possible link between the most important electrolytes and BK contraction needs to be understood. Weindberg et al. (1997) suggested that BK-induced contraction in mesenteric arterial rings is indirect, through activation of BK2 receptors, resulting in liberation of prostanoids from outside the endothelium. They conclude that thromboxane A2 was probably an intermediate in this response but they didn't exclude the participation of other prostanoids. Jensen and Conlon (1997) stated that the involvement of arachidonic acid metabolites and 5-hydroxytryptaminergic nerves in the mechanism of action of BK was possible. Pang and Knox (1997) were the first to demonstrate that BK could induce cyclooxygenase (COX)-2.

They suggested that conversion of increased arachidonic acid release to PGE2 by COX-1 is mainly involved in short-term effect, whereas BK2 receptor-related COX-2 induction is important in the long-term PGE2 release. Pang and Knox 1998 concluded that BK caused cyclooxygenase induction and prostaglandin E2 release in human airway smooth muscle cells. An understanding of the relationship between BK and the arachidonic acid pathway is important.

Austin et al 1996, concluded that the actions of BK in the human nasal airway are accounted for by the release of histamine. Histamine is an important mediator of bronchoconstriction that is released from mast cells. Histamine constricts smooth muscle by binding to H1 receptors. It also increases the production of prostaglandins and as already discussed, prostaglandins affect smooth muscle. As histamine has both a direct effect and an indirect effect, via prostaglandins, on smooth muscle the possibility of one of the histamine receptors mediating the BK response should be explored.

2. REVIEW OF LITERATURE

2.1 GALLBLADDER

2.1.1 Gallbladder Motility

The control of gallbladder motility is complex and it depends on an intricate interplay of neural and hormonal factors. In addition to well-known agonist effects and adrenergic relaxation, neurally mediated non-adrenergic non-cholinergic relaxation in human gallbladder muscle has been demonstrated (McKirdy et al. 1994ii). Some inflammatory mediators, e.g. Prostacyclin, can reduce gallbladder contractility (Kotall et al. 1984). Motility defects may play a role in the pathogenesis of gallstones (Patankar et al. 1995). Therefore an understanding of the relationship between inflammatory mediators and the control mechanisms of gallbladder contraction is crucial to the understanding of the mechanism of gallstone formation. Cabrini and Calixto (1997) reported that BK induces graded contraction in guinea-pig gallbladder in vitro though activation of BK2 receptors and prostanoid release and that activation of the BK1 receptor causes a weak contraction. The inflammatory mediator BK causes contraction of guinea pig gallbladder, possibly involving a third BK receptor (Cabrini et al. 1995).

When food is digested in the gastrointestinal tract, the gallbladder will begin to empty. The emptying of the gallbladder increases as fatty-food enters the duodenum. The emptying is a

result of rhythmic contractions of the wall of the gallbladder, but effective emptying also requires simultaneous relaxation of the sphincter of Oddi that guards the exit of the common bile duct into the duodenum. The potent stimulus for causing the gallbladder contractions is the hormone cholecystokinin (CCK). However, in addition to CCK, the gallbladder is also stimulated less strongly by cholinergic nerve fibres from both the vagi and the enteric nervous system. These are the same nerves that promote motility and secretion in other parts of the upper gastrointestinal tract.

Even with relatively strong contractions of the gallbladder, emptying can still be difficult because the sphincter of Oddi normally remains tonically contracted. Therefore, before emptying will occur, it must be relaxed. At least three factors help in this; (i) CCK, instead of stimulating the sphincter of Oddi, has a weak relaxing effect, but this effect is usually not sufficient by itself to allow significant emptying. (ii) the rhythmic contractions of the gallbladder are associated with peristaltic waves in the sphincter of Oddi, causing a leading wave of relaxation that partially inhibits the sphincter in advance of the peristaltic wave. (iii) When intestinal peristaltic waves travel over the wall of the duodenum itself, the relaxation phase of each of these waves strongly relaxes the sphincter of Oddi along with the relaxation of the muscle of the gut wall. This seems to be by far the most potent of all the relaxant effects on the sphincter of Oddi. As a result, bile usually enters the duodenum in the form of squirts that are synchronized with the duodenal peristaltic contractions.

2.1.2 Gallstone Formation

Bile salts are formed in the hepatic cells from cholesterol, and in the process of secreting the bile salts, about one-tenth as much cholesterol is also generated into the bile. The precursor of bile salts is cholesterol, which is supplied in the diet or synthesised in the liver cells during the course of fat metabolism then converted to colic acid or chenodeoxycholic acid. These acids then combine principally with glycine and to a lesser extent with taurine to form glyco-conjugated and tauro-conjugated bile acids. The salts of these acids are secreted in the bile. The bile salts have two important actions in the intestinal tract. First, they have a detergent action on the fat particles in the food, which decreases the surface tension of the particles and allows the agitation in the intestinal tract to break the fat globules into minute sizes. This is called the emulsifying of bile salts. Second, bile salts help in the absorption of fatty acids, monoglycerides, cholesterol and other lipids from the intestinal tract.

When the bile becomes concentrated in the gallbladder, the bile salts and lecithin become concentrated along with the cholesterol, which keeps the cholesterol in solution. Under abnormal conditions the cholesterol may precipitate, resulting in the formation of gallstones. The different conditions that can cause cholesterol precipitation are (i) too much absorption of water from the bile, (ii) too little secretion of bile salts and lecithin in the bile, (iii) too much secretion of cholesterol in the bile, (iv) inflammation of the epithelium of the gallbladder and reduced gallbladder emptying, leading to prolonged retention of bile in the gallbaldder (Patankar et al. 1995).

As a result, of some or all of these mechanisms, cholesterol begins to precipitate, usually forming many crystals of cholesterol on the surface of the inflamed mucosa or on small precipitated particles of bilirubin. These, in turn, act as point for further precipitation of cholesterol, and the crystals grow larger. Occasionally tremendous numbers of sand like stones develop, but much more frequently these coalesce to form large gallstones, or even a single stone that fills the entire gallbladder. (Blitzer et al. 1982)

2.2 KININS

2.2.1 Overview

Kallikreins are a group of serine proteases that are found in glandular cells, neutrophils and biological fluids. The history of the kallikrein system can be traced back to the observations of Abelous and Bardier in 1909 that intravenous injection into the dog of an alcohol-insoluble fraction of human urine caused a pronounced, but reversible, fall in systolic blood pressure (Abelous and Bardier in 1909). However it was not until twenty years later when Frey (1926) and Frey and Kraut (1928) found that human urine contained a thermolabile, nondialyzable substance that, on intravenous injection, caused a prolonged arterial hypotension in the dog. The kallikreins are divided into two main groups: tissue and plasma kallikreins (Fiedler 1979), they are inactive but readily activated. They are chemically similar but not identical. All kallikreins were first thought to originate in the pancreas. We now know that all kallikreins do not originate from a single parent organ e.g. urinary kallikrein originates from the kidney.

The two types, tissue and plasma, differ in their molecular weight, substrate specificity, immunological characteristics, type of kinin released (Webster and Pierce 1960, Webster 1970, Fritz et al. 1977) and functional importance (Bhoola et al. 1979). A single gene codes for plasma kallikrein (Seidah et al. 1989), whereas tissue kallikrein is a member of a multi-gene family that shows different patterns of tissue-specific gene expression (Mason et al. 1983, Qin et al. 1991).

The vasoactive peptides, Kinins are released by kallikreins from endogenous substrates called kininogens, by means of enzymatic action (Muller-Esterl 1989). Enzymes that possess the capacity to release kinins from kininogens are collectively called kininogenases, a term first introduced by Werle (1960). This generic name includes enzymes such as plasma and tissue kallikreins, trypsin and plasmin. So the primary function of tissue kallikreins is to form kinins but an additional function is processing enzymes for protein precursor molecules, enzymes and hormones (Lazure et al. 1983, Seidah et al. 1988).

As stated previously, the kallikrein-kinin system consists of plasma and tissue kallkreins. The plasma kallikreins are expressed mainly in the liver (Clemens et al. 1994) but they can be synthesised and stored by endothelial and vascular smooth muscle cells (Schmaier et al. 1988, Figueroa et al. 1992) and they differ from tissue kallikrein, which are encoded in various genes and expressed in a variety of cells (Clemens 1994, Yu at al. 1998, Abdalla et al 2000). Plasmatic high molecular weight kininogens (HMWK) and tissue low molecular weight kininogens (LMWK) derive from a single gene which by alternative splicing yields two mRNAs that differ in size (3.5 vs. 1.7kb) and function (Schmaier 1997). The system in plasma

is considered to be a constitutive, anti-coagulant that protects the endothelium (Linz et al. 1995). The system in tissue is an essential factor in the tissue reaction to noxious or artificial stimuli and in tissue repair. As figure 2 (page 25) shows, HMWK is the precursor of BK while LMWK generates kallidin both of which are further transformed into fragments which display specific biological activities (Regoli and Barabe 1980). There are three pathways that lead to the production of kinins – the two mentioned above (plasma dependant pathway, tissue dependant pathway) and a plasma/tissue independent pathway.

To say that kinins are more important as patho-physiological rather than as physiological mediators might be risky but is probably true. Gagninella and Kachur 1989 concluded that BK released through the action of plasma kallikrein, can dilate small blood vessels, increase their permeability and induce pain. In this way BK can bring about the warmth, redness and swelling of inflammatory lesions. Lembeck and Grisebacher (1996) suggest both a physiological and patho-physiological role for kinin in the pancreas. Kinins exist in insects, reptiles, amphibians and mammals (Erdos 1979). Bradykinin and kallidin are the predominant kinins with effects on the gastrointestinal tract of mammals. Although the role of kinins in normal physiology is not clearly defined, they act as mediators in inflammation (Marceau et al. 1983. Couture et al. 2001). In nanomolar concentration, kinins increase smooth muscle contraction, increase vascular permeability, produce oedema, evoke nociception and induce electrolyte secretion. Hyperkininemia, or excessive tissue production of kinins, is associated with hypotension and carcinoid flushing (Oates et al. 1964), postgastrectomy dumping syndrome (Wong et al. 1974), haemorrhagic pancreatitis (Ofstad 1970), allergic rhinitis (Proud et al. 1983), and intestinal mucosal inflammation (Zeitlin 1970). At the cellular level,

kinins promote chloride and glucose transport, release known transmitters from neurones, directly or indirectly activate phospholipase A_2 and phospholipase C and stimulate osteoclasts. Receptors linked to specific G protein-couples second messengers mediate the cellular actions of kinins.

Bradykinin and kallidin are the predominant kinins with effects on the GI tract of mammals. BK is a potent inflammatory peptide whose generation in tissues and body fluids elicits numerous responses including vasodilation, oedema, smooth muscle spasm, as well as pain and hyperalgesia. There is substantial evidence that BK and related kinins contribute to the inflammatory response in acute and chronic diseases including reactions, arthritis, asthma, sepsis, and inflammatory bowel diseases (Proud et al. 1983). As, BK appears such an important factor in both normal and pathological conditions this thesis focuses on the action of BK on normal and on stone-bearing human gallbladder smooth muscle *in vitro*.

2.2.2 Historical Background

As mentioned in the previous section, a reference to a substance resembling glandular kallikrein appeared in the publications of Abelous and Bardier (1909). They showed that an alcohol-insoluble fraction of human urine caused hypotension when injected intravenously into the anaesthetised dog. Pribram and Hernheiser (1920) observed a similar hypotensive action of human urine on the blood pressure of the rabbit. As described in the previous section, Frey and Kraut (1928) also demonstrated the presence of this substance in human urine. The compound responsible for the hypotensive property was isolated and shown to be a

nondialysable, thermolabile substance of high molecular weight (Frey and Kraut 1928). As a result of this Werle et al. (1934) extensively studied this biologically active material, and in a series of publications demonstrated its presence in blood, pancreas and the salivary glands (Frey et al. 1932, 1950). They assumed that the hypotensive element in urine had originated from the pancreas and named it kallikrein (Werle 1934), from kallikreas, the Greek word for pancreas. This name proved to be unfortunate because nowadays we know that many kallikreins exist and although they are all serine proteases with a chemical similarity, they are not identical and they do not all originate from the one organ e.g. urinary kallikrein originates from the kidney.

In 1937, Werle et al. demonstrated that when the kallikreins were incubated with serum they enzymically released a smooth muscle contracting substance from an inactive precursor, kininogen. They named the new biologically active molecule, substance DK (Werle and Grunz 1939), from darmkontrahierende substanz. Unlike the kallkreins, it was dialysable and thermostable, contracted the isolated guinea pig ileum and showed marked hypotensive activity. This demonstration of the release of DK by kallikrein preceded by two years the analogous demonstration that renin owed its hypertensive action to the release of a precursor peptide from a substrate in plasma. In 1948, Werle and Berek renamed substance DK and called it kallidin and named the precursor, the substrate for kallikrein, kallidinogen. About this time, Rocha-Silva and colleagues (1949) reported that incubation of snake venom extracts of *B. jararaca* or trypsin with the globulin fraction of dog plasma resulted in the formation of a substance that produced a slow, delayed contraction of the isolated guinea pig ileum. The response interval for this new substance was seven times greater than that obtained with

histamine or acetycholine. The authors called it bradykinin, 'brady' - meaning slow and 'kinin' meaning movement. The two systems, the trypsin- or snake venom-bradykininogen-bradykinin and the kallikrein-kallidinogen-kallidin system are very similar.

A number of peptides closely resembling kallidin and BK in their pharmacological properties have since been discovered in insect venoms. Discovery of the first nonmammalian kinin in wasp venom was made by Jaques and Schacter in 1954. They drew attention to the similarity of the contractile responses of the isolated guinea pig ileum to kallidin, BK and an extract of wasp venom, assayed in the presence of inhibitors to acetylcholine and histamine (Schachter 1960). After further studies, Schacter and Thain (1954) gave these substances the generic name of kinins. At the end of the 1950's, Bhoola observed the presence of a kinin-like peptide in the venom of hornets which, in addition, contained high concentrations of acetycholine, 5-HT and histamine (Bhoola et al. 1960).

2.2.3 Actions

Kinins are small peptides that have diverse biological affects. The two mammalian kinins, kallidin and BK, influence the cardinal features of inflammation as well as a number of cellular functions, including blood pressure and local blood flow, electrolyte and glucose transport, and cell proliferation. Concentrations of kinins in the nanomolar or sub-nanomolar range induce intestinal smooth muscle contraction and evoke mucosal electrolyte secretion. Kinins exert their biological effects through activation of two transmembrane G-protein coupled receptors – BK1 and BK2. The cellular actions of kinins are modified by their ability

to stimulate the release of many second-generation mediators, e.g., platelet-activating factor, leukotrienes, prostaglandins. substance P (neurogenic inflammation), acetylcholine, and noradrenaline (sympathetic nerves). Kinins also stimulate the secretion of renin from the kidney (Beierwaltes et al. 1985), release vasopressin from the neurohypophysis (Baertschi et al. 1981), and stimulate secretion of catecholamines from the adrenal medulla (Staszewska-Barazak and Vane 1967).

The actions of BK vary between species but also between tissues of the same species. The work in thesis focuses on the action of BK on normal and stone-bearing gallbladder smooth muscle tissue and will be discussed in more detail later.

2.2.3.1 Kinins

Kinins contract extravascular, isolated smooth muscle preparations but relax the rat duodenum (Werle and Berek 1948). Apparently, several smooth muscle preparations have been reported to respond directly to tissue kallikrein, in the absence of added kininogen (Frey et al. 1950). Beraldo et al. (1966) made a similar observation on the isolated rat uterus and suggested that the contractions were due to a direct combination of tissue kallikrein with receptors on the muscle. The mechanism of this action is now clarified and the contraction is due to the release of kinin from kininogen molecules present within the uterine muscle (Figueiredo et al. 1990).

Isolated airway smooth muscle of most species, including humans and guinea pig, shows a biphasic response to kinin peptides. At concentrations of 10^{-8} M, the relaxations are more

prominent, whereas at higher concentrations only contractions are observed (Bhoola et al. 1962). Removal of the epithelium abolishes the relaxation phase, believed to be mediated by PGE_2. When PGE_2 is released it binds to cell surface receptors to stimulate intracellular cAMP synthesis. At high concentrations both prostaglandin-dependent and prostaglandin-independent contractions are observed. Bradykinin is a potent stimulant of bronchial C-fibres, and some of its actions are mediated through the release of sensory neuropeptides from capsaicin-sensitive nerves (Kaufman et al. 1980). Multiple mechanisms are probably involved in the *in vivo* bronchoconstrictor action of BK, namely (a) release of prostanoids, thromboxanes, substance P, and neuropeptides; (b) C-fibre-evoked axon reflexes, and (c) direct stimulation.

The occurrence of increased amounts of BK in cardiac muscle after acute myocardial infarction is considered to produce beneficial effects by reducing or limiting the size of the myocardial infarction. Coronary infusion of BK causes improved cardiac function and metabolism, reduces reperfusion injury and prevents arrhythmias in isolated ischaemic hearts. The specific action of BK on smooth muscle is discussed later.

Kinins are considered to play a primary role in inflammation (Bathon and Proud 1991). If kinins are injected into human skin, all the cardinal signs and symptoms of inflammation are observed. Initially, there is pain due to direct stimulation of sensory C-fibre terminals and then the release of substance P, which adds to the neurogenic inflammation. As an axon reflex-mediated flare follows this is caused by local vasodilatation and oedema created by the increase in vascular permeability and extravasation of proteins and fluid. Cytokines are then

released from monocytes and this attracts leucocytes to the injection site. This release of cytokines is kinin stimulated. The ability of kinins to release the cytokines - IL-1 and TNF (Tiffany and Burch 1989) and many second-generation mediators of which prostaglandins and leukotrienes are formed most frequently, is of great importance. Prostaglandins were reported as necessary for the full manifestation of the cardiovascular response to BK (Stebbins et al. 1985).

2.2.3.2 Bradykinin

Bradykinin plays important roles in tissue inflammation and mediation of pain (Bathon and Proud 1991). Inflammatory pain is thought to be initiated by chemical mediators released from damaged tissue that stimulate specific receptor sites on nocieptive sensory neurones. Bradykinin has long been thought to be a mediator of inflammation (Steranak et al. 1987) and is also involved in the mediation of pain and hyperalgesia caused by irritant substances in many animal models, based on the increased presence of BK in injured tissue (Garcia-Leme 1978). It is known that BK receptors are localised to sensory neurones and that a variety of BK receptor antagonists selectively elicit analgesia in several models of tissue damage.

Nociceptive neural transmission involves small-diameter un-myelinated or thinly myelinated fibres with small cell bodies in the dorsal spinal cord. By contrast, touch and pressure sensation is conveyed by thick myelinated fibres with large cell bodies in the dorsal root ganglion and terminals in relatively deep layers of the dorsal spinal cord. BK receptors are selectively localised to sites involved in nociception : superficial layers of the spinal cord, thin

un-myelinated fibres and small neuronal cells in sensory ganglion while nociceptive : sensory fibres terminate in trigeminal ganglion cells, such fibres from the heart fully traverse the stellate ganglion, whose major cells are sympathetic (White 1957). This pattern fits with BK receptor localisation to small cells in the stellate ganglion. Peripheral terminals of primary afferents contain the BK receptors that respond directly to tissue injury and inflammation.

Bradykinin (BK) and kallidin appear to act on tissue in a similar manner but BK has been more intensively studied. Both have similar pharmacological potency in causing vasodilation and increasing vascular permeability that result in local oedema (Bhoola and Schachter 1960, Carter et al. 1974). At first, there is separation of endothelial cells, resulting in extravasation of fluid from the capillaries. The early observations of Bhoola et al. (1960) on the capillary permeability effect of plasma kallikrein in the guinea pig skin have been shown to be due to the generation of kinins (Imamura et al. 1984). Locally injected kinins increase vascular permeability and paw volume in the guinea pig and rabbit pre-treated with KII inhibitor, with a decreasing order of potency :

Kinins > BK > kallidin > Met-Lys-bradykinin > des-Arg^9-Bradykinin
(Whalley et al. 1987i).

Enhanced vascular permeability arising from formation of kinins in cancerous tissue may have profound consequences for the metastatic spread of tumours. Kinin (Bradykinin and Hyp^3-Bradykinin) levels in ascitic fluid from patients with ovarian, gastric, and hepatic carcinomas are sufficiently increased to cause enhanced vascular permeability (Matsumara et al

1988,1990). BK has been increasingly implicated in the aetiology of a number of pain conditions and it is generally true that both BK agonist activity and BK receptor numbers increase under such conditions.

Kinins are the most potent pain-producing substances when applied to a blister base (Armstrong et al 1957) or when injected intradermally (Ferreira 1972). It has long been known that BK causes acute pain following application to a blister base and after an intra-arterial injection in laboratory animals (Bhoola et al 1962). Bradykinin has been described as the most potent endogenous algogenic substance (Steranka et al. 1987). The painful sensation has a latency of 30 to 120 seconds and persists for at least 75 to 150 seconds, depending on the concentration used (Bhoola et al. 1962, Bhoola and Schacter 1960). When compared to 5-HT, BK is at least 10 times more potent in causing pain on the blister base (Whalley et al. 1987ii). The algesic action of BK is potentiated by thromboxanes, prostaglandins, and 5-HT.

Kinins evoke pain by stimulating nociceptive afferent nerves (Juan and Lembeck 1974). Formalin injected subcutaneously into the rat paw causes prolonged activation of neurones receiving sensory information from that area. Formalin injected into the paw fails to fire the dorsal horn neurones when the BK receptors are desensitised or if added in the presence of a kinin antagonist (Haley et al. 1989). Kinins generated in injured or inflamed tissues activate sensory receptors that relay nociceptive information, through C and Aδ afferent fibres to the substantia gelatinosa of the spinal cord.

The resurgence of interest in kinin receptors as potential drug targets and the development of potent antagonists has led to further evaluation of some of the kinin-associated cellular actions. Their role in inflammation was reviewed by Marceau 1983, Burch et al. 1989 and Schwaninger et al. 1999. Kinins act as mitogens, stimulating DNA synthesis and thereby promoting cell proliferation (Marceau and Tremblay 1986, Whitfield et al. 1970). The ability of kinins to induce cell division could enhance the spread of cancerous cells and increase the proliferation of epidermal cells in disorders such as psoriasis. This is an area in need of much new research effort.

2.3 BRADYKININ

2.3.1 Background

Bradykinin and the closely related peptide kallidin are vasoactive peptides formed by the action of enzymes on protein substrates termed, kininogens. As mentioned previously, Werle and Berek 1948 described kallikrein, an enzyme in urine, which acts on serine proteins to liberate kallidin, which causes a fall in blood pressure. Rocha-Silva et al. (1949) showed that the snake venom, *B. jararaca,* when incubated with serum, gave rise to a substance that caused hypotension but which also caused a slow contraction of certain smooth muscle preparations (isolated guinea pig iluem). As this was 'slow' and 'moving' it was called 'brady' 'kinin'. Bradykinin is the most well studied kinin. It is a vasoactive peptide formed by the action of enzymes on protein substrates termed kininogens.

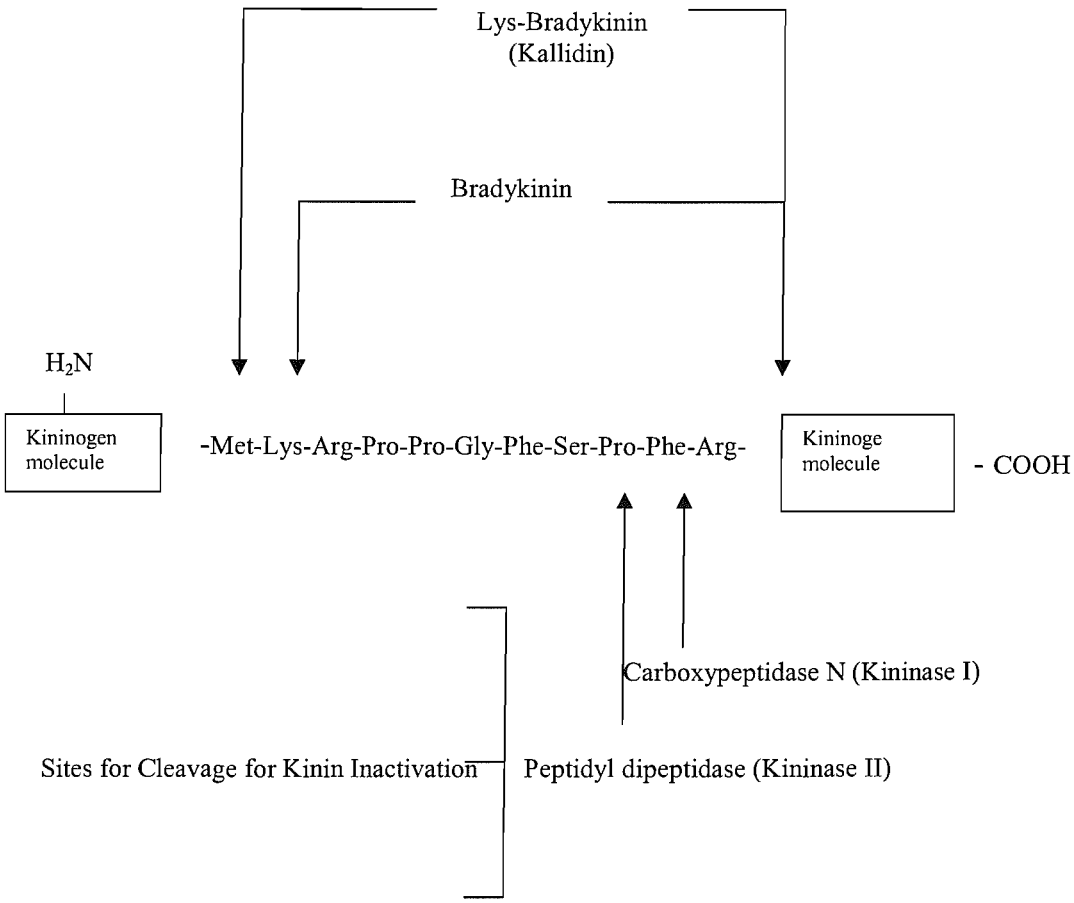


Fig 1. The amino acid structure of Bradykinin - Bradykinin is a nonapeptide : The sites of proteolytic cleavage for formation of kallidin and BK are shown in the upper half of the figure. The sites of cleavage for BK inactivation are shown in the lower half of the figure. (Rang and Dale1991)

The formation of BK is discussed in detail in section 2.3.2 but in short, the kinins have two protein precursors. High molecular weight kininogen comprises 20% of circulating

kininogens, and is acted on by the enzyme plasma kallikrein, yielding BK, whilst the action of tissue kallikrein on low molecular weight kininogen (80% of circulating kininogen) generates kallidin, a BK homologue. It is thought that the low molecular weight of the kallidin precursor allows it to pass freely from the vasculature into tissue. Both are regulated by high levels of circulating inhibitors e.g. α-antiprotease. The main enzymes, which inactivate BK, are called kininases. Bradykinin's action is terminated by enzymatic cleavage. Angiostensin converting enzyme is bound to vascular membranes in the pulmonary capillary bed and elsewhere, and inactivates the vast majority of plasma BK. Carboxypeptidase N, a soluble plasma enzyme, gives BK a 15-second half-life. One of the kininases, kininase II, is the same as angiotensin-converting enzyme. This is a peptidyl dipeptidase, which removes the two C-terminal amino acids from the kinin.

2.3.2 BK Formation

The plasma kinin forming system consists of three essential plasma proteins that interact in a complex fashion once bound to certain negatively charged inorganic surfaces, macromolecular complexes formed during an inflammation response, or bound to proteins along cell surfaces. The three factors are (i) coagulation factor XII (hageman Factor or HF), (ii) prekallikrein, and (iii) high molecular weight kininogen.

Factor XII is activated by contact with surfaces having a negative charge, such as collagen, bacterial lipopolysaccharides, urate crystals etc. Factor XIIa results and it in turn converts plasma prekallikrein to kallikrein and kallikrein digests high molecular weight kininogen to

liberate BK (Fujikawa and McMullen 1983). Kallikrein can also activate the complement system and can convert plasminogen to plasmin. Factor XIIa has a second substrate in plasma, namely, coagulation factor XI. Activation of surface-bound Factor XI by Factor XIIa initiates the intrinsic coagulation cascade. Thus, the interactions of all four of these proteins are known as 'contact activation' (Colman 1980, Schaimer 1998), and the formation of BK is therefore a cleavage product of the initiating step of this cascade. (Ginsberg et al. 1980). There is also a tissue pathway by which BK is generated. There is intracellular conversion of a prokallikrein to tissue kallikrein by enzymes that are as yet not well characterised and tissue kallikrein is secreted into the local milieu. There it digests low molecular weight kininogen (LK) to generate lysyl-bradykinin (kallidin) and a plasmin aminopeptidase converts kallidin to BK. The constituents of the plasma kinin-forming (Factor XII, Prekallikrein etc.) cascade are bound along the surface of cells, such as endothelial cells, and neutrophils, and activation of the system may occur along the cell surface.

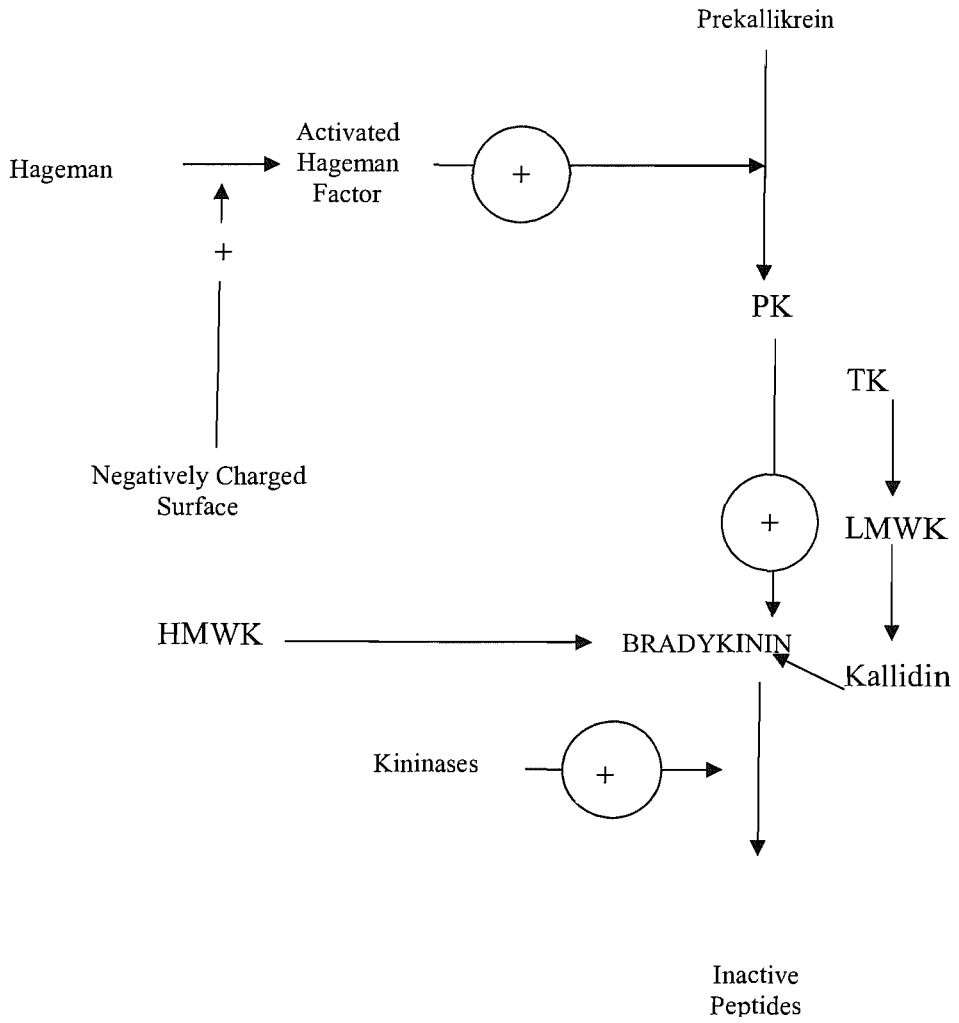


Fig 2. Formation and breakdown of Bradykinin. HMWK = high molecular kininogen. LMWK = low molecular kininogen. PK = Plasma Kallikrein. TK = Tissue Kallikrein. This substance probably acts as a substrate for kallikrein and as a cofactor in the activation of prekallikrein. (Rang and Dale 1991).

2.3.3 Receptor Types

The effects produced by BK, including those on nociceptive neurones, are due to activation of specific BK receptors. There is a lot of evidence for at least two types of BK receptors, BK1 and BK2, and both have been extensively studied over the last decade. Several pharmacological experiments suggest further classes of BK receptors, such as BK3 in the lung (Farmer et al. 1989ii) and BK4 and BK5 receptors in the oesophagus (Saha et al. 1991). Various studies have suggested receptor heterogeneity in other tissues as well (Regoli et al 1994, Regoli et al 1993). Cabrini et al. (1995) and Field (1992) suggested that BK induced contraction of the guinea pig gallbladder may involve a third BK receptor.

At least five receptors have been reported. Early classification was based on agonist potency and receptor heterogeneity exists from a diverse pharmacological profile in which different analogues are responsible for different actions of the parent molecule. Now for receptor characterisation, there is an initial requirement of structure-activity studies to establish an order of potency of agonists, identification of differences in activity profile, measurement of the affinity of competitive antagonists, and mechanisms involved in signal transduction. So far, two types of kinin receptors have been characterised, BK1 and BK2 (Regoli and Barabe 1980, Vavrek and Stewart 1985, Haddad et al. 2000), initially they were classified based on the affinity for their agonists and antagonists (Regoli and Barabe 1980). This pharmacological kinin-receptor classification was later confirmed by the cloning of two separate genes coding for the BK2 and BK1 receptor (Menke et al. 1994, McEachern et al. 1991). The human BK1

and BK2 receptor genes have been co-localised on the same part of the chromosome 14 (Powell et al. 1993, Bachvarov et al. 1998).

Kinin receptors were first classified according to relative potencies of kinin agonists on isolated smooth muscle preparations. The des-Arg^9-Bradykinin metabolite showed a much greater affinity for contracting the rabbit aorta, mesenteric vein, and basilar artery than did either of the two standard kinins (Regoli et al 1977, Whalley et al 1983). A thousand-fold greater concentration of the parent kinin was needed than des-Arg^9-BK to achieve similar potency on the rabbit aorta (BK1 receptor). A study was conducted to determine whether des-Arg-kinins produced relaxation of isolated vessels. Both des-Arg^9-BK and BK produced a dose-dependent relaxation of isolated rabbit superior mesentric arteries. The results indicate that in addition to vasoconstriction des-Arg^9-BK can produce vasorelaxation, which may be mediated through stimulation of BK1 receptors and the subsequent release of prostaglandins. Des-Arg^9-BK produced relaxation and contraction of the rabbit mesenteric artery precontracted with phenylephrine, the relaxation was mediated by prostaglandins, as the relaxation response to both BK and des-Arg^9-BK was inhibited by the cyclo-oxygenase inhibitor indomethacin, and the contraction was a direct effect (Churchill and Ward 1986,1987). Stimulation of BK1 receptors on macrophages released IL-1 and TNF (Tiffany and Burch 1989). Whereas the increase in protein synthesis, collagen building and cell division in human foetal lung fibroblasts by des-Arg^9-Bradykinin was inhibited by des-Arg^9-Leu^8-Bradykinin (BK1 antagonist), the formation of prostaglandins by BK was unaffected.

Some large arteries and veins (e.g. aorta and the anterior mesenteric vein) as well as some perpheral vascular beds (e.g. the coronary vessels) have the ability of generating BK1 receptors while other organs (e.g. the external jugular vein) have not or have very little, and the BK1 receptors seem to be absent. The reason for this phenomenon remains unclear. BK1 receptors seemed to be absent normally, but expression in smooth muscle cells and fibroblasts becomes evident in pathological states, particularly in inflammation or after exposure of tissue to noxious stimuli. When rabbits were injected with lipo-polysaccharide, a hypotensive effect was observed with des-Arg^9-Bradykinin (Regoli et al. 1981). The intravenous injection of 10 micrograms of lipo-polysaccharide extracted from E.coli leads to the appearance of a hypotensive effect for des-Arg^9-BK and increased significantly the vasodilatory effect of the peptide in isolated hearts and its contractile effects in strips of large arteries and veins. Induction of BK1 receptors was observed in the cardiovascular responses of rabbits when KII-ACE inhibitors were injected (Nwator and Whalley 1989) and the spontaneous increase in contractile responses to des-Arg^9-BK of rabbit aortic strips incubated in vitro was noted (DeBlois et al. 1991). The enhanced sensitivity of isolated tissues containing BK1 receptors increased with time in parallel with the specific binding of ^{3}H-des-Arg^9-Bradykinin. Inhibition of BK1 receptor induction by cycloheximide or actinomycin-D has led to the proposal of de novo synthesis of BK1 receptors in some tissues as a result of inflammation or tissue trauma (Regoli and Barabe 1980, Regoli et al. 1981). In fact, following an intravenous injection of *Escherichia coli* endotoxin in the rabbit, des-Arg^9-Bradykinin produced pronounced hypotension, an effect not observed in control animals. IL-1 has been proposed as the endogenous trigger for the induction of BK1 receptors and the mediator that enhances the effects of des-Arg^9-Bradykinin in inflammation (DeBlois et al 1991).

Most of the actions of BK and kallidin are mediated through the BK2 receptor, which essentially does not respond to des-Arg^9-Bradykinin or -kallidin. On most membranes, the Kd for the BK2 receptor is in the range 0.7 to 5 nm. In contrast to the rabbit aorta (a primary site for the BKI receptors), the muscle relaxant actions of kinins on the dog carotid artery and bladder (Regoli et al 1986) and isolated rabbit mesentery and human basilar arteries are effected through BK2 receptors (Whalley et al. 1987, Nwator and Whalley 1989), as is the venocontractile action of BK on the rabbit jugular vein. Analysis of the potency of cyclical kinins, Met-Lys-bradykinin, kallidin, and BK, in increasing paw volume and skin vascular permeability suggested species differences in BK2 receptors mediating these actions in the rat when compared to the guinea pig and rabbit, even though the involvement of the BK1 receptor was excluded for all three species (Whalley 1987). Various claims have been made regarding further classes of BK receptors Farmer and colleagues (1989ii) proposed the existence of a BK3 receptor in the airways. The claim for a third BK receptor remains controversial. Many studies have purported to demonstrate heterogeneity among BK2 receptors and BK3 and BK4 (Farmer et al. 1989, Saha et al. 1991, Regoli et al. 1993, and Cabrini et al. 1995) have been reported. The discovery of more selective and potent antagonists will contribute to our understanding of kinin receptor pharmacology.

The most important difference between the BK1 and BK2 receptor is that the BK2 is constitutively expressed and activated by the parent molecule, while the BK1 receptor, which is generally underexpressed in normal tissue i.e it is hardly detectable under physiological conditions (Marceau et al. 1998), is often induced under inflammatory conditions. In addition, in contrast, to the BK2 receptor, the BK1 receptor does not desensitise (Austin et al 1997) and

is up-regulated by its own agonist (Schanstra et al. 1998, Tschope 2000) or after treatment with bacterial endotoxins or cytokines such as interleukin beta and TNF-alpha. These observations suggest that under inflammatory conditions, the BK1 receptor may superimpose its activity to that of the BK2 receptor. The BK1 receptor may exert a strategic role in inflammatory diseases with an immune component (diabetes, rheumatois arthritis and multiple sclerosis).

Many studies have purported to demonstrate heterogeneity among BK2 receptors (Burch et al. 1990) and several authors (Farmer et al. 1989, Saha et al 1990, Saha et al 1991) have reported that further classes of BK receptors exist (as mentioned above). Most of these studies have utilised the weak first generation BK antagonists. Thus, the results of many investigations remain suspect. However, certain studies do present persuasive evidence for receptor heterogeneity. In certain smooth muscle preparations there is evidence for smooth muscle and neuronal receptors. In rat deferens, BK elicited contraction by a direct action on smooth muscle and potentiated neurogenic contractions by modulating prejunctionally on the sympathetic nerve endings (Llona et al. 1987). There was no evidence for participation by BK1 receptors.

Most actions of BK, including the acute activation of nociceptors and the production of pain, are mediated through the BK2 receptor (Levine et al. 1993), Dray et al. 1992). A variety of peptide analogues have been produced that act as selective antagonists at the BK1 and BK2 receptor, so enabling this receptor to be comprehensively studied and characterised. These substances have been critical for establishing a physiological role for BK as well as

identifying some of its pathophysiological effects. The BK2 receptor has been cloned and its sequence of 364 amino acids shows a high degree of homology between a number of species, including humans (McEachern et al. 1991, Hess et al. 1992). This receptor belongs to a superfamily of receptors whose members all have seven membrane spanning domains and are coupled with a G protein. A number of sites have been identified which are important for BK binding and for receptor regulation.

As mentioned previously, it has been reported that BK1 receptors are induced during inflammatory conditions (Regoli and Barabe 1980) whereas BK2 receptors are synthesised constitutively. However, in cultured bovine pulmonary artery endothelial cells, Smith et al. (1995) have shown that both BK1 and BK2 receptors are made constitutively.

Our understanding of BK receptors is increasing very rapidly with the advent of specific antagonists and the cloning of the receptor proteins and related signal transduction proteins. The availability of antagonists is allowing us to appreciate how important endogenous kinins are in the pathology of inflammation and pain.

2.3.4 Functions/Actions

Bradykinin has been reported to stimulate a variety of extracellular responses and intracellular events - such as hypotension, bronchoconstriction, epithelial secretion, increase in vascular permeability, connective tissue proliferation and gastrointestinal, uterine contractions and activation of phospholipase A_2 to augment arachidonic acid metabolism (Regoli et al. 1990,

Regoli and Barabe 1980) . BK may also serve as a neurotransmitter in the central and peripheral nervous system (Steranka et al 1988, Fujiwara et al 1989). BK can act directly on the target cells or its effects may be mediated by prostaglandins and leukotrienes released from target or neighbouring cells, endothelium derived-relaxing factors released from endothelium, histamine released from mast cells and neurotransmitters released from motor and sensory nerves (Johnson and Erdos 1973, Ueda et al. 1984, Burch et al. 1990). In some tissues BK acts both directly and indirectly to produce complex responses (Maggi et al. 1989, Regoli et al 1990, Griesbacher et al. 1989).

In plasma, BK is first digested by carboxpeptidase N (also known as anaphylatoxin inactivator (Erdos and Sloane 1962), which removes the C-terminal- Arg leaving des-Arg^{9}-bradykinin. This peptide lacks the inflammatory function of BK (vasodilation, increased permeability) that is evident in skin or smooth muscle, but can interact with B1 receptors in the vasculature to cause hypotension.

In gastrointestinal smooth muscles (rabbit duodenum, rat duodenum and colon, guinea pig ileum and stomach) BK produces a biphasic response consisting of a brief relaxation followed by contraction (Griesbacher et al. 1989). The inhibitory effect of BK is thought to be caused by a direct action on the muscle (Hall and Bonta 1973) whereas the excitatory effect may involve a direct action as well as indirect actions involving prostaglandins and the release of neurotransmitters (Walker and Wilson 1979, Saha et al. 1990).

Studies have shown that acute gallbladder inflammation increases endogenous BK-stimulated prostglandins (PG) release and inhibits guinea-pig gallbladder contractility. Data also suggest that the enhanced BK-stimulated PG release seen in acutely inflamed guinea-pig gallbladder is due to the synthesis of cyclo-oxygenase-1 and prostacyclin synthase (Bogar et al. 1999). The experiment used tissue slices from guines pigs that were anesthetized and underwent common bile duct ligation. The slices were oxygenated in various tissue culture mediums at 37^0C. The mediums were then assayed for net release of prostaglandins, thromboxanes and leukotrienes

2.3.5 Mechanism of Action

BK nociceptors have been localised to nociceptive pathways (sensory nerve terminals and small cells in dorsal root ganglia by autoradiography of ^{3}H-BK (Schill et al. 1989). In keeping with this, excitation of nociceptors by BK has been demonstrated in a number of circumstances. These range from direct recording of C-fibre nociceptor activity *in vitro* and BK elicited nociceptive reflexes (Langet et al. 1990), to recording BK mediated excitation of nociceptive receptors in vivo in skin, skeletal muscle, joints and visceral organs (Foch and Mense 1976, Sato et al. 1989). In most cases, the BK1 agonist des-Arg^9-BK was ineffective, and antagonists to the BK2 but not BK1 receptor blocked the acute activation of sensory neurones by BK (Haley et al 1989).

Various approaches have been used to elucidate the receptor-coupled mechanisms of nociceptor activation. Because of the difficulties of studying fine un-myelinated nociceptive fibres in vivo these studies have relied heavily on the use of tissues and cells maintained in

vitro. For example clonal cell lines such as the neuroblastoma-glioma (Higastida and Brown 1987) hybrid respond to BK and have been used to correlate the changes in membrane ion conductance with intracellular biochemical events. In these cells BK produces a biphasic response. Initially there is a transient hyperpolarization due to the action of Ca^{2+} sensitive K^+ conductance initiated by the release of Ca^{2+} from intracellular stores following the formation of inositol 1,4,5-trisphosphate (IP3). This hyperpolarization is followed by a prolonged depolarisation due to inhibition of the voltage-dependent K^+ conductance.

Sensory neurones respond somewhat differently to BK, but some similarities are evident. Thus, BK produces an immediate depolarisation of sensory neurones and nociceptive fibres that is directly related to an increase in membrane permeability, mainly to Na^+ (Dray et al. 1992). Membrane excitability is also altered due to effects on a number of cellular messenger systems. For example, BK stimulates membrane phospholipase C to generate diacylglycerol (DAG) and IP3, which activate intracellular protein kinase C (PKC) and elevate intracellular Ca^{2+}, respectively (Dray et al 1992, McGuirk and Dolphin 1992). These events may also be associated with cell activation. However IP3-mediated release of intracellular Ca^{2+} appears less important in sensory neurones than in other cell types. Stimulation of PKC has also been shown to inhibit membrane Ca^{2+} conductance (Boland et al. 1991) but the relationship between this and increased cellular excitability is less clear, although it is possible that a Ca^{2+} dependent K^+ conductance may be altered. The BK-induced changes in sensory neuronal excitability are coupled to a G protein since the effects were reduced by GDP-β-S and prolonged by GTP-γ–S, even though neural activation by BK was insensitive to pertussis toxin (McGuirk and Dolphin 1992). BK may also stimulate the production of arachidonic acid to be

derived from the breakdown of diacylglycerol DAG (Gammon et al. 1989). Arachidonic acid oxygenation products were, for many years, held to be synonymous with prostaglandins, but more recently they have been shown to include thromboxane A_2 and multiple lipoxygenase pathway products, most notably the leukotrienes. It is unclear whether arachidonic acid acts by itself upon release or whether it is metabolised further to form prostanoids. However, it is well known that BK can directly activate membrane phospholipase A2 (Bhoola et al. 1992, Farmer and Burch 1992) in many cell types, leading to the production of prostanoids via subsequent cyclo-oxygenase and lipoxygenase activity. In visceral sensory neurones, BK indirectly increases excitability via the activation of cAMP and the subsequent inhibition of Ca^{2+} dependent K^+ permeability, which underlies a post-spike hyperpolarization. This action of BK is abolished by indomethacin and mimicked by prostaglandins, suggesting that it is mediated indirectly via prostanoid production (Weinreich 1986).

The activation of sensory fibres by BK also causes the release of neuropeptides such as substance P, neurokinin A (nA) and calcitonin gene-related peptide (CGRP) (Geppetti et al. 1990). These peptides can also mediate a number of local pro-inflammatory effects and contribute to nociceptor sensitisation and hyperalgesia. Substance P and CGRP stimulate vascular endothelial cells to release the smooth muscle relaxant nitric oxide. Substance P contracts endothelium and allows the leakage of plasma protein that accounts for neurogenic oedema. Substance P and nA also induce degranulation of mast cells and the release of histamine, which is a pro-inflammatory mediator. As BK itself is also a powerful inflammatory mediator, it is likely that it has major algesic and pro-inflammatory influences.

2.4 AIMS

As described previously, it has been reported that BK has a variety of effects on different tissue and various BK receptors mediate this action. It is well documented that two classes of BK exist but further classes of BK receptors have been reported. It has also been suggested that a different population of BK receptors exist under normal physiological and under pathological conditions. Prior to the experiments in this thesis the full effects of BK on human gallbladder tissue was not known. The aim behind the initial set of experiments in this thesis was

- to explore the effects of BK on human gallbladder tissue.
- to investigate the possible receptors that mediate this action.
- note any differences in BK receptor population, between normal and stone-bearing gallbladder tissue.
- to investigate any mechanisim that may effect the BK action.

As a result of these investigations a fuller picture of BK contraction of gallbladder smooth may be understand.

3. MATERIALS AND METHODS

3.1 Introduction

The first task of this project was to establish a protocol for the *in vitro* investigation of the action of BK on gallbladder smooth muscle tissue. This section of the thesis outlines the procedure for obtaining the sample tissue in the operating theatre and the *in vitro* technique used.

As an aside to the main study a number of patients, who later had their gallbladder strips used in the *in vitro* study, had their circulating levels of cholecystokinin (CCK) measured prior to surgery. Blood (6ml) was taken for CCK radioimmunoassay, from fasting individuals and then again at 15, 30, 45 and 60 minutes, after ingestion of a standard fatty meal (23.8g of fat per 100g meal). Ultrasound measurement of gallbladder volume, using the ellipsoid formula, was performed at five minute intervals for 30 minutes and at 10 minute intervals thereafter up to 1 hour. As a result a small number of individuals then will have three sets of data: CCK assay results, gallbladder ultrasound recordings (both prior to surgery) and *in vitro* gallbladder contractility data.

3.2 Disclaimer and Constent

The procedure used for the work described in this thesis was carried out in conjunction with Mr N. W. Pearce (MD candidate, University of Southampton). Mr Pearce was involved in the

initial setting up of the contractility work and we jointly obtained some very initial data. Any overlap in the description of the materials or methods used is a result of such collaboration (Pearce et al 1999). No contractility data presented in this thesis was obtained solely by Mr Pearce. The CCK assay was previously developed in the laboratory (Bailey 1992). The ultrasound procedure was performed by Mr N. W. Pearce. Patients consent for using blood samples for assay techniques was obtained. Ethical approval for tissue sampling and the use of blood for assays was obtained from the South and West Hampshire local research ethics committee.

3.3 The *in vitro* investigation

3.3.1 Tissue type

The gallbladder smooth muscle cells vary considerable in size but are typically 100-300μm long and 2-5μm in width. Cells characteristically narrow toward their ends but may be irregular in cross-section. Juxtaposed cells exhibit a variety of junctions that serve as sites of communications linkages. They are also embedded in a connective tissue matrix, which is a product of the cell's synthetic and secretory activities. This matrix limits distension of the tissue to lengths near the optimum for force development.

The gallbladder tissue for these experiments was obtained from two groups of patients. Stone bearing gallbladders were obtained from patients undergoing cholecystectomy for gallstone disease. The majority of cholecystectomies for gallstones were performed laparoscopically,

but a number were carried out using the open or "mini" technique. Healthy gallbladders were removed from patients undergoing extensive resections for pancreatic or hepatic disease. Certain patients and certain types of tissue were excluded as only two groups of tissue were required for the study i.e. stone-bearing and normal (non stone-bearing) with no other gallbladder abnormalities. Therefore, patients undergoing cholecystectomy for acute cholecystitis; gallbladders that appeared macroscopically acutely inflamed at the time of surgery; gallbladders that were grossly fibrotic and gallbladders that were severely traumatised during operation were excluded. Gallbladders that were initially obtained for the normal group but which, when opened, were found to contain stones were transferred into the gallstone group. Complete obstruction of the cystic duct (mucocoele) or common bile duct (obstructive jaundice) were also exclusion criteria in view of their potential to distort subsequent contractility in vitro.

3.3.2 Krebs Bicarbonate Solution

Five litres of Krebs solution of the following composition was made daily:
118.4 mM NaCl, 25 mM $NaHCO_3$, 11.7 mM glucose 4.7 mM KCL, 1.9 mM $CaCl_2$, 1.2 mM $KHPO_4$ and 1.2 mM $MgSO_4$. The solution was made up in distilled water and then mixed by placing it on a magnetic stirrer for 5 minutes. About 50mls of this solution was poured into a container which would be used to transport the tissue for theatre. All of the solution was then stored at 4^oC until used. Any remaining solution was discarded at the end of the day.

3.3.3 Preparation of BK and Other Peptides

Bradykinin, des-Arg^9-Leu^8-BK, des-Arg^9-BK, carbachol, HOE-140 and cholecystokinin (CCK) (Sigma, Poole, UK) were all made up as 10 mM stock solutions with distilled water and 1ml aliquots were frozen at -20^{o}C and thawed as required. After thawing, 100 μL, BK, CCK and des-Arg^9-bradykinin stock solutions were diluted with Krebs solution to give a concentration of 100μM. The antagonists mentioned in section 3.3.7 were made up on a daily basis as needed. Each was diluted with distilled water according to the concentration required (as indicated section 3.3.7). All diluted solutions were kept at 4^{o}C during the course of the experiments.

3.3.4 Experimental Protocol

A full thickness segment from the anterior aspect of the body of the gallbladder was removed, in theatre, and placed immediately in Krebs solution on ice. The tissue was transported to the laboratory, 3 to 6 muscle strips (10-15mm X 2-3mm) from the anterior aspect of the fundus of the gallbladder were cut in a longitudinal direction. The strips were then suspended, in Krebs solution, in an organ bath (Linton, UK, see dig) at 37^{0}C, gassed with 5% CO_2 in oxygen. When available, the times of ligation of the cystic artery, removal of the gallbladder from the patient, of its immersion in Krebs solution and of the placement of individual strips in the organ bath were all recorded.

Each strip was attached by size 20 fishing hooks and cotton thread at either end, to an isotonic transducer (Harvard apparatus, UK) under a 1g pre-load. The signals from the transducers were fed into a custom made amplifier interface which was connected to a Macintosh Apple computer loaded with Maclab/8e chart. The Maclab/8e chart system is an integrated system of hardware and software, designed to record, display and analyse experimental data. 8e v3.5 chart, together with the Maclab hardware and a Macintosh computer give the capabilities of a multi-channel chart recorder but also provides a range of features that transcend the limitations of a mechanical pen and ink recorder. This gave a continuous recording of spontaneous activity and response to stimuli as millivolts (mV) in the range –200 to +200mV. An example of a typical strip can be noted in Fig 3 below.

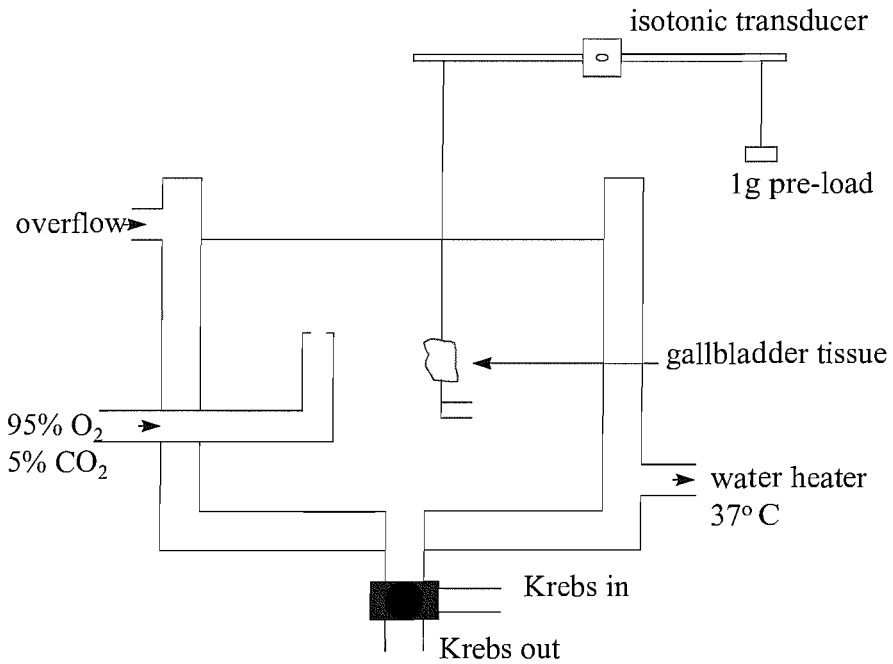


Fig 3. A single organ bath. Typically six of these were used in any one experiment.

3.3.5 The organ bath and transducer

Quantitative estimates of the mechanical output of the muscles have provided important inferences about the mechanism of chemomechanical transduction and its control mechanisms. The measurement of muscle contraction also provides a way of assessing the effects of assessing the effects of neurotransmitters, drugs and hormones. The important mechanical variables are force, length and the derived variable of shortening velocity (length/time). The basic approach in muscle mechanics is to control all of these factors except the measured dependent variable. Such measurements employ muscle which the cells are aligned the axis in which force and length are determined. A curve generated in which the length of the muscle is fixed and the force is measured during a contraction is called isometric. The other possible measurement characterising the output of a muscle and used in this project, is obtained when the load or stress on a muscle is held constant (see fig. 3) and the shortening velocity is measured, this is isotonic measurement.

A viable tissue was allowed to equilibrate in the organ bath until a continuous baseline appeared. Whatever the mV reading was at this stage it was recorded and used as the baseline for that particular strip (e.g. 1.2mV, see fig 4 below). Various different baseline recording would be obtained for each strip. The first dose of the agonist would then be added e.g. 1uM BK and the strip would contract and a new mV recorded when the curve reached a peak (40.2mV). The next dose would be added and the peak again recorded (e.g. 2^{nd} dose peak, 95.6mV). The final dose (in this example) would then be added and the peak recorded 159mV.

The maximum dose readings in this example would be 157.8mV (159 – 1.2) and this would be taken as 100% maximum contraction for this particular strip and all dose result would be as a percentage of this maximum contraction.

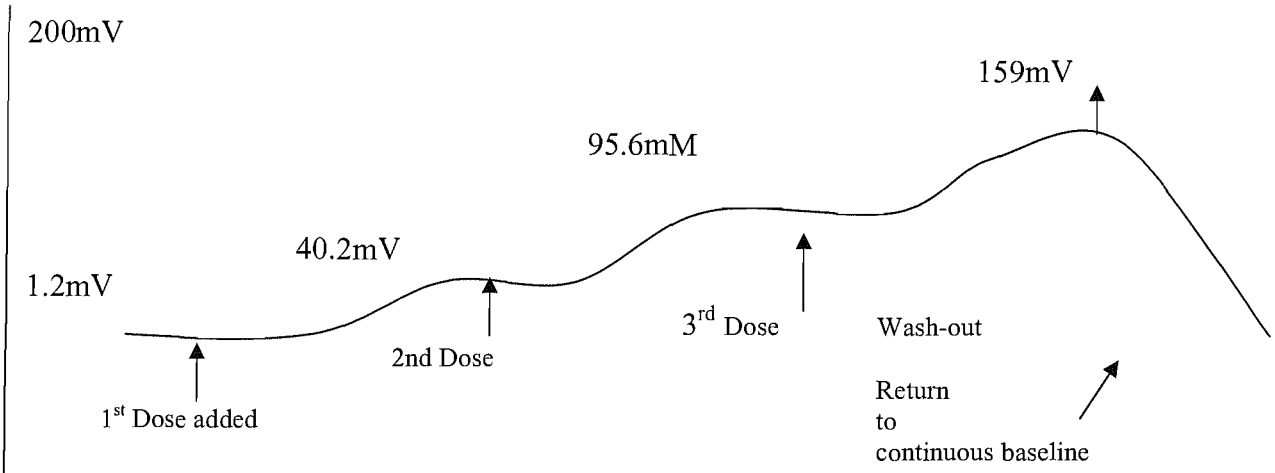


Fig 4. A typical trace from the output of a transducer connected to a muscle strip.

3.3.6 Viability Testing

The time of placement in the organ bath was recorded and the strips were left to equilibrate for two hours, after which viability was assessed with carbachol 100μmol. Those strips that did not contract i.e. no activity recorded in millivolts, in response to this standard stimulus were discarded. Viable strips were then washed out and irrigated with Krebs solution and allowed to equilibrate until a steady baseline was achieved, before proceeding with the experiment. Strips that failed to return to a steady baseline were also discarded

3.3.7 Generation of a Concentration Response Curve

The concentration response curve (CRC) is the standard method of assessing the contractile response of human tissue to hormonal or chemical stimuli in vitro. Throughout these experiments a standard technique has been used for producing CRCs.

The initial aim was to determine the range of concentrations of BK required to obtain concentration response curves in human gallbladder strips *in vitro* and to identify if this response was repeatable. To generate a CRC, BK was added incrementally, starting with 10μl of the dilute solution and going up to a cumulative total of 1ml, giving a concentration in the organ bath between 1-100μ.M. In total seven different concentrations of BK were used for the CRC : 0.1,1,5,10,20,50 and 100μ.M. At each concentration the contraction was allowed to reach a plateau before the level of contraction was recorded and prior to addition of further BK. If no contraction was observed at a particular concentration within 3 minutes, then the next increment of BK was added. After achieving the maximum concentration within the organ bath and recording the peak contraction the strips were washed out and irrigated with Krebs solution until a steady baseline had returned prior to proceeding with any further part of the experiment.

The majority of viable strips exhibit one of two typical baseline patterns prior to stimulation. These were either a low frequency relatively high amplitude contraction or a fine high frequency contraction. These contractions were usually abolished by the addition of BK or

other stimuli. For consistency and simplicity of calculation the mid point of these small contractions was taken as the baseline value.

Viable strips would produce a consistent repeatable CRC for BK up to 4 times over a period of 6 hours whilst in the organ bath. Tissue death was marked by any one of following patterns after washout and irrigation; persistent contraction, loss of normal tone and subsequent relaxation markedly below the previous baseline or bizarre irregular high amplitude contractions. Strips displaying any of these patterns were deemed non-viable and not subjected to further stimuli.

3.3.8 The *in vitro* study

The study of BK receptor antagonists has lead to the characterisation of two classes of BK receptors, as noted previously. The BK1 receptors exhibit a higher affinity for the kinin metabolites - des-Arg^{9}-BK and des-Arg^{10}-Kallidin are selectively and competitively antagonised by the BK1 receptor antagonists - des-Arg^{9}-Leu^{8}-BK or des-Arg^{10}-BK. In contrast to BK1 receptors the BK2 receptors exhibit a high affinity for BK and kallidin but a low affinity for the BK1 agonist des-Arg^{9}-BK. The range of BK CRCs and the optimum concentration of each antagonist were established from previous literature. All previous work was in agreement that the doses used in these experiments which provided maximal receptor blockade. The BK CRC was repeated, sequentially, in the presence of one or more the following antagonists.

- des-Arg^9-Leu^8-BK (1μM) - a selective BK1 receptor antagonist.
- HOE-140 (1μM) - a selective BK2 receptor antagonist.
- both receptor antagonists combined (1μM des-Arg^9-Leu^8-BK, 1μM HOE-140).
- mepyramine (1μM) - a H1-histamine receptor antagonist.
- atropine (1μM) - a muscarinic receptor antagonist.
- prazosin (0.1μM) - a alpha1- adrenoceptor blocker.
- tetrodotoxin (0.03μM) - a Na channel blocker.
- quinidine (1μM) - a K channel blocker.
- indomethacin (3μM) - a cyclo-oxygenase inhibitor.
- ryanodine (10μM) - blocks intracellular Ca release.
- the response was also tested in Ca free medium
- SR27897 (0.5nM) - a CCK-A receptor antagonist

In a separate set of experiments the concentration of des-Arg^9-Leu^8-BK and HOE-140 was progressively increased (1-25μM and 1-100μM respectively) and a constant amount of BK was added (10μM). The BK concentration response curve was also repeated with different concentrations of des-Arg^9-Leu^8-BK (100,500nM) and HOE-140 (30,100nM). A BK1 receptor agonist, des-Arg^9-BK (0.1-20μM; Sigma, UK) dose response curve was obtained in the presence of each BK receptor antagonist (des-Arg^9-Leu^8-BK (1μM) and HOE-140 (1μM). A range of concentrations for a CCK dose response was obtained using the incremental procedure as described in section 3.3.6. (CCK was added (0.1-10μM)). The CCK response curve (10nM-1μM) was repeated in the presence of the BK receptor antagonists, i.e

- des-Arg^9-Leu^8-BK (1μM) - a selective BK1 receptor antagonist.
- HOE-140 (1μM) - a selective BK2 receptor antagonist.
- both receptor antagonists combined (1μM des-Arg^9-Leu^8-BK, 1μM HOE-140).

3.3.9 Statistical Analysis

The response in each curve was calculated as a percentage of the maximum response obtained in the original curve, for that particular gallbladder muscle strip. Points on the figures are presented as mean ± SEM. All data were analyzed by one-way analysis of variance (ANOVA) which was used to test for statistical significance. The F ratio was computed from the ratio of the mean sum of squared deviations of each group's mean from the overall mean (weighted by the size of the group) and the mean sum of the squared deviations of each item from the item's group mean. A, P value of <0.05 was taken as significant. Thus if the null hypothesis is correct (i.e. no significance) the F value will be about 1 and a large F value indicates an 'effect' and the P value to be less than 0.05

The ED_{50} values relate to the concentration of the agonist required to elicit 50% response relative to the maximum response of the agonist been tested.

$_pK_B$ values (-log K_B) were obtained according to the formula:

$$K_B = [A] / [CR - 1]$$

where [A] is the concentration of the antagonist and [CR] is the concentration ratio, which is the ED_{50} in the presence of the antagonist, divided by the ED_{50} in the absence of the antagonist.

3.4 Measurement of CCK

3.4.1 Patients

Gallstone patients who were awaiting elective cholecystectomy for symptomatic cholelithasis were recruited into the study. Patients with greater than 50% of gallbladder volume occupied by stones were excluded.

3.4.2 Study Protocol

Patients were fasted for six hours prior to the study. The gallbladder volume was measured sonographically using the ellipsoid formula (length x width x height x 0.52). Blood (6ml) was taken for CCK radioimmunoassay. The patients ate a standard fatty meal (yorkie bar, 29g of saturated fat in a 52g bar, Nestle UK) and gallbladder volume was measured at 5 minute intervals for 30 minutes, and at 10 minute intervals thereafter up to 1 hour. Further blood samples for CCK assay were taken at 15, 30, 45 and 60 minutes after the test meal. The blood was placed into 2 ice cold vacutainers (3ml in each) containing 5μM EDTA. Samples were stored on ice and centrifuged (2500rpm at 4^0C for 12 minutes) within 30 minutes of collection.

Plasma from each blood sample was placed into a polystyrene tube and stored at -20^{0}C until extraction.

3.4.3 CCK assay

The samples were processed and analysed as previously validated in the laboratory (Bailey 1992). Plasma samples were thawed and 1ml of plasma was mixed with 2ml 98% ethanol, vortexed for 10 seconds and centrifuged at 3000rpm for 15 minutes. The supernatant was poured into a polystyrene tube and the sample dried overnight in a rotary evaporator. The dried extract of each 1ml of plasma was stored at -20^{0}C until reconstituted in assay buffer prior to assay.

The assay buffer consisted of 1 litre of distilled water with 9.6g Na2.2H20, 0.825g KH2P04, 3.72g Na2EDTA, 500mg Azide and 2.5g gelatin equilibrated to pH 7.4. Anti-CCK-8 was developed in rabbit using synthetic sulfated CCK-8, conjugated to KLH, as the immunogen. 200µl of this dried antiserum (Sigma, UK) was reconstituted in 20ml of assay buffer, divided into 2.5ml aliquots and stored at -20^{0}C. 370kBq of Bolton Hunter I^{125} CCK-8 (specific activity 2000ci/mmol, Amersham UK) was reconstituted in 500µl of distilled water and 20µl aliquots were stored as stock solutions at -20^{0}C. For incubation in the assay, tracer stock solutions were diluted in assay buffer to give assay tracer activity of 1000-1500cpm per 400µl. Dried ethanol extracts of lml plasma were reconstituted in 500µl of assay buffer.

The total assay volume consisted of lml per tube, containing 500µl reconstituted plasma extract, 100µl anti-serum and 400µl I^{125}-labelled CCK-8 with 1000-1500cpm as the total tracer activity. Tubes were set up at room temperature and reagents added at room temperature. CCK-8 standards (125pM-0.49pM) were prepared by serial dilution of stock solutions in assay buffer. Each concentration was incubated in duplicate. The tubes were capped and incubated at 4^0C for 72 hours.

After incubation free and bound tracer were separated. NORIT activated charcoal (0.8g) and Dextrab garde C (0.08g) were added to 50ml of cols assay buffer and mixed in an ice bath for 20 minutes. 300µl of charcoal mixture was added to each tube with all tubes in a cold water tray at 4^0C. After addition of charcoal the tubes were centrifuged for 10 minutes, 3000 rpm at 4^0C and the supernatant was separated from the pellet. The supernatant (bound) was counted for 10 minutes on an automated gamma counter.

3.4.4 Statistical analysis

Data are presented as mean and $\pm$ SEM. Tests for statistical significance were performed using t-test. A P value of < 0.05 was taken as significant.

3.5. *In vivo* V *In vitro* study

The data from the individuals in the studies were examined. It was noted that a number of patients from each study had ultrasound recordings, CCK assay data and also CCK and/or BK *in vitro* gallbladder contraction data. This data from the studies were complied and examined.

4. RESULTS

This section may be divided into three different sections. The first part will detail the initial bradykinin (BK) receptor work on both normal and stone-bearing gallbladder tissue. The second part accounts for the examination of other mechanisms that may affect or mediate the BK action. The final part examines the *in vivo* V *in vitro* argument - what conclusions can be drawn from the laboratory experiments when compared to what happens within the living body. Do the results achieved in the laboratory replicate what happens *in vitro* ?

4.1 Bradykinin and bradykinin Receptor activity

4.1.1 Bradykinin dose response curves
4.1.2 Bradykinin receptor antagonists
4.1.3 Responses in normal and in the presence of gallstones
4.1.4 BK1 and BK2 receptors combined
4.1.5 Bradykinin 1 receptor agonist
4.1.6 Maximum BK response
4.1.7 Viability testing

4.2 Independent mechanisms of BK contraction

4.2.1 Cholecystokinin

4.2.1.1 CCK dose response curve and BK receptor interaction
4.2.1.2 CCK receptors
4.2.1.3 Possible BK and CCK receptor interaction

4.2.2 Various other possible receptors, channel mediators

4.3 *In vivo* V *In vitro* argument

4.1 Bradykinin and bradykinin Receptor activity

4.1.1 Bradykinin dose response curves

Initially a range of concentrations for a BK dose-response curve needed to be determined. BK was incrementally added from 10^{-9}M to 10^{-3}M. As the table shows below, no strip demonstrated a contraction of greater than 1% of the maximum at a concentration below 10^{-7}M. A contraction within 2% of the recorded maximum was reached in all strips at 10^{-4} and the mean for all five strips showed a contraction of 99.5% of the maximum. Therefore, all subsequent BK dose response curves were obtained using the range 10^{-7}M to 10^{-4}M.

BK (M)	10^{-9}	10^{-8}	10^{-7}	10^{-6}	$5x10^{-6}$	10^{-5}	$2x10^{-5}$	$5x10^{-4}$	10^{-4}	10^{-3}
A1	0	0	1.1	4.5	4.5	23.7	45.1	73.8	99.9	100
A2	0	0	0.8	2.5	9.2	17.3	28.5	62.7	100	98
A3	0.4	0.1	9.3	61.9	81.2	91	93.9	99.7	98.8	100
A4	0	0	6.5	35.3	47	56	70	82.5	98.7	100
A5	1	1	6.8	24.8	45.3	60.8	73	82.3	100	99.1
Mean	**0.3**	**0.2**	**4.9**	**25.8**	**37.4**	**49.8**	**62.1**	**80.2**	**99.5**	**99.4**

Table 4.1. Contraction of human gallbladder strips *in vitro* in response to Bradykinin (BK) in the range 1n-1mM, expressed as a % of the maximum contraction. The five strips are from three different gallbladders.

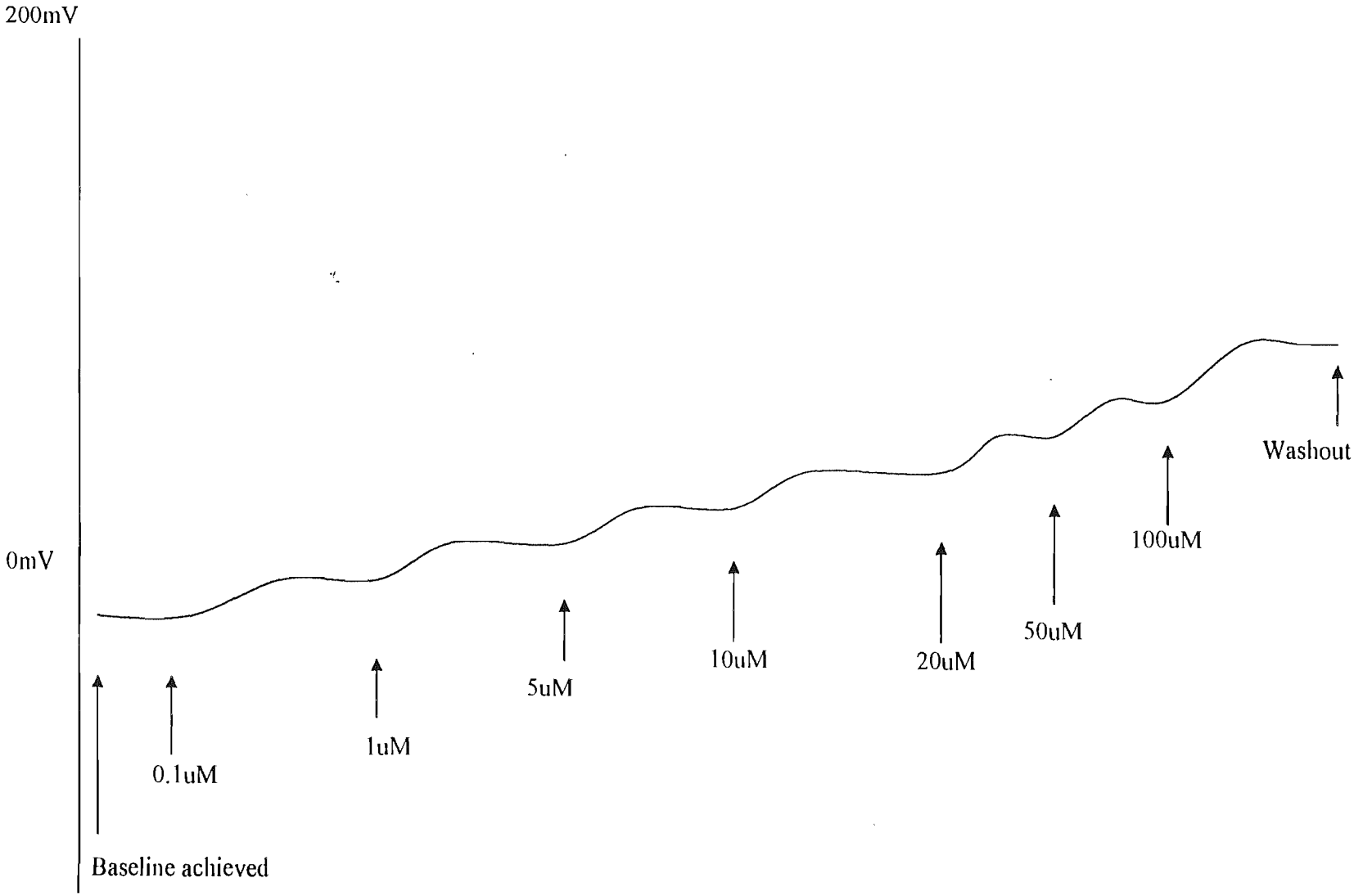
As described in the materials and methods section, cumulative response curves were use i.e. each dose was added as the preceding dose had reached a peak. The trace on the following page (CRC trace) clearly illustrates how the concentration curves were achieved for a BK cumulative dosing.

When a steady baseline was achieved the first dose would be added i.e. 0.1μM. The strip's contraction would be replicated by the transducer where an increase in mV would be noted. Typically up to six of these tracings would be in each experiment corresponding to each strip. An example of the output can be seen in table 4.2 below. The final mV reading is taken as the maximum contraction and each dose is calculated as a percentage of that maximum, for example strip one below contracts from -168.2mV to -68.0mV. This is a maximum contraction of 100.2mV. The addition of 0.1μM achieves a contraction of 13.1mV; this is 13.7% of maximum contraction.

BK (μM)	0	0.1	1.0	5.0	10.0	20.0	50.0	100.0
strip1	-168.2	-155.1	-134.3	-105.9	-94.8	-87.2	-70.6	-68.0
strip2	89.5	95.1	101.3	115.6	125	130	154.	166.8
strip3	-97.7	-97	-95.8	91	-89.5	-87	-85.5	-85.1

Table 4.2. Contraction of three gallbladder strips *in vitro* in response to Bradykinin (BK). The data is expressed in milli-volts. The three strips are from the one gallbladder.

After the final dose is added and a peak response achieved a washout occurs and the strips return to the original base-line and the next dose curve can begin when a steady base-line occurs.



CRC trace – A concentration response trace, achieved for BK 10^{-7}M to 10^{-4}M.

Both normal and stone-bearing gallbladder strips were subjected to the concentration response curve, previously obtained, both showed similar and repeatable concentration response curves to BK. ED_{50} = 4.9 & 5.5μM (fig 4.1). The BK curve appears to be still increasing at the final concentration this is because the final point on each curve is indeed the maximum contraction (i.e. 100%) achieved at that dose (i.e. 100μM). So although the curves appear to be steeply climbing at the highest dose, rather than approaching a plateau, this is because the maximum contraction is always achieved at the maximum dose, this is also the case for all subsequent figures.

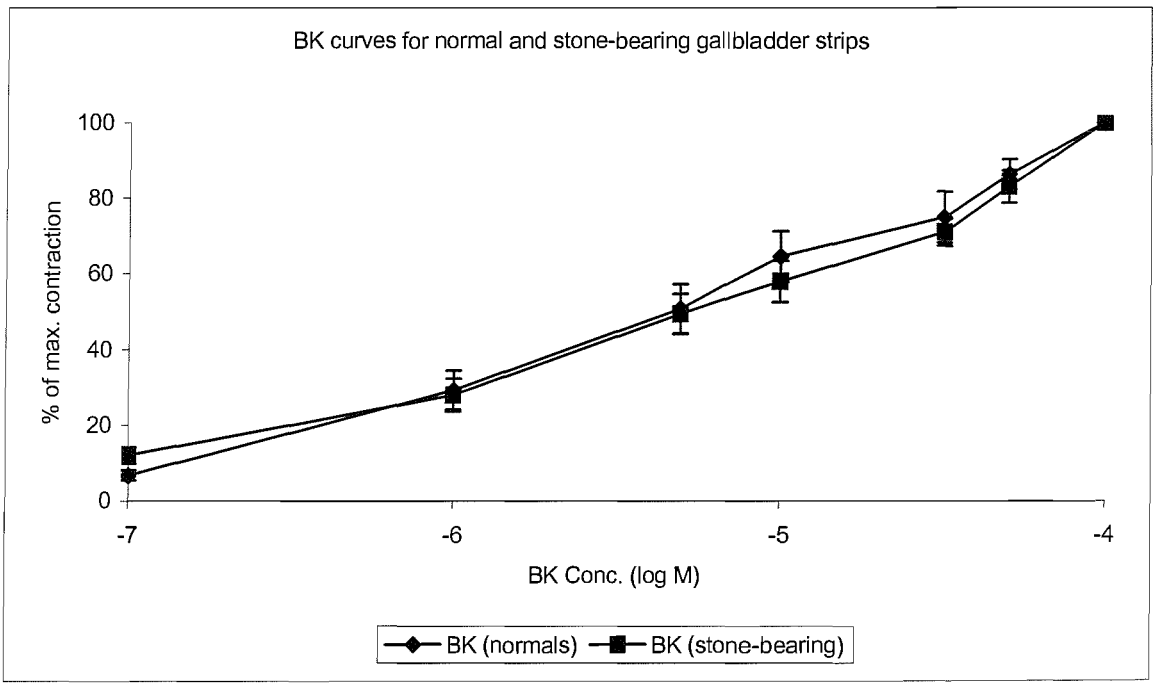


Fig 4.1 Bradykinin concentration response curves for normal and stone-bearing gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E of 5-6 experiments. The ED_{50} values obtained for normal and stone-bearing are 4.9μM and 5.5μM respectively.

4.1.2 Bradykinin receptor antagonists

The optimum concentration for each BK receptor antagonist needed to be established. The concentration of each receptor antagonist was progressively increased and a constant amount of BK was added. When the concentration of des-Arg^9-Leu^8- BK or HOE-140 was increased no difference was noted in the response to BK (10μM), (fig 4.2). Therefore, 1μM of each antagonist was recognised as a sufficient concentration for BK1 and BK2 receptor blockade.

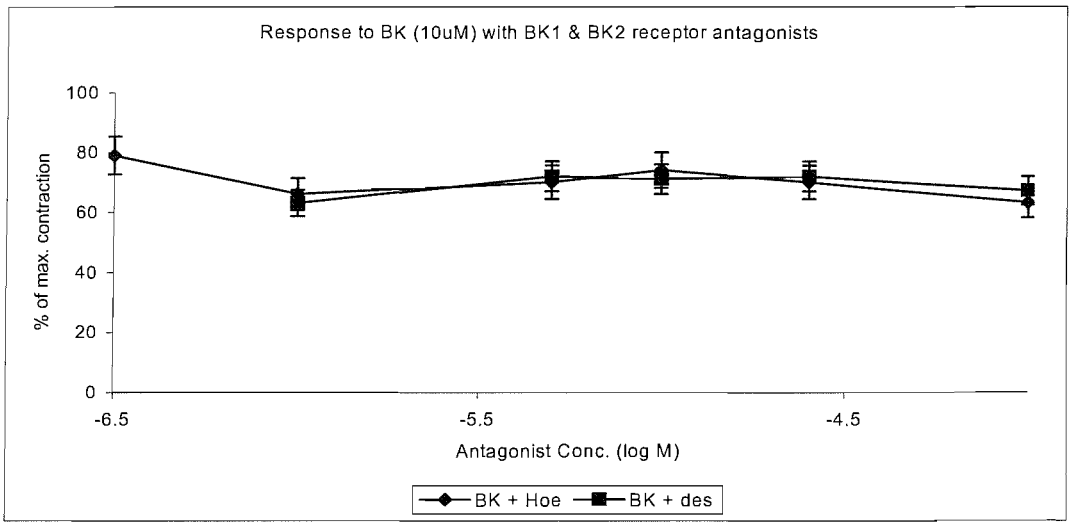


Fig 4.2 The response to BK (10uM) with varying concentrations of the BK1 and BK2 receptor antagonists. Various human gallbladder strips were exposed to 10μM of BK and the contraction was noted and used as the maximum. After washout the same amount of BK was added with different concentrations of the BK1 receptor antagonist (1, 5, 10, 25, 50μM). After each point (1-50μM) the strip was washed out. Various other gallbladder strips were exposed to 10μM of BK and the BK2 receptor antagonist concentration was varied – 0.5, 1, 5, 10, 20, 50μM. No significant difference was noted in the response to increasing amounts of each receptor antagonist concentration, so 1μM was recognised as a sufficient concentration for both receptor blockades. Each point above represents the mean ± S.E. of 4-5 experiments.

4.1.3 Responses in normal tissue and in the presence of gallstones

The BK receptor in normal and stone-bearing gallbladder tissue was examined. When the BK1 receptor was blocked, by the introduction of des-Arg^9-Leu^8- BK (1μM), a significant rightward shift (ANOVA P=0.001, F=13.42) of the curve was observed in normal gallbladder

strips (fig 4.3) but not in stone-bearing gallbladder strips (ANOVA P=0.64,F=0.043). ED_{50}= 31.5 & 9.8μM. BK2 receptor blockade, using HOE- 140 (1μM), produced a significant rightward shift of the curve in normal and stone-bearing gallbladder strips (fig 4.5 & 4.6). ED_{50} = 37.2 & 29.4μM; ANOVA P=0.001 & 0.002, F=19.63 & 17.880) respectively.

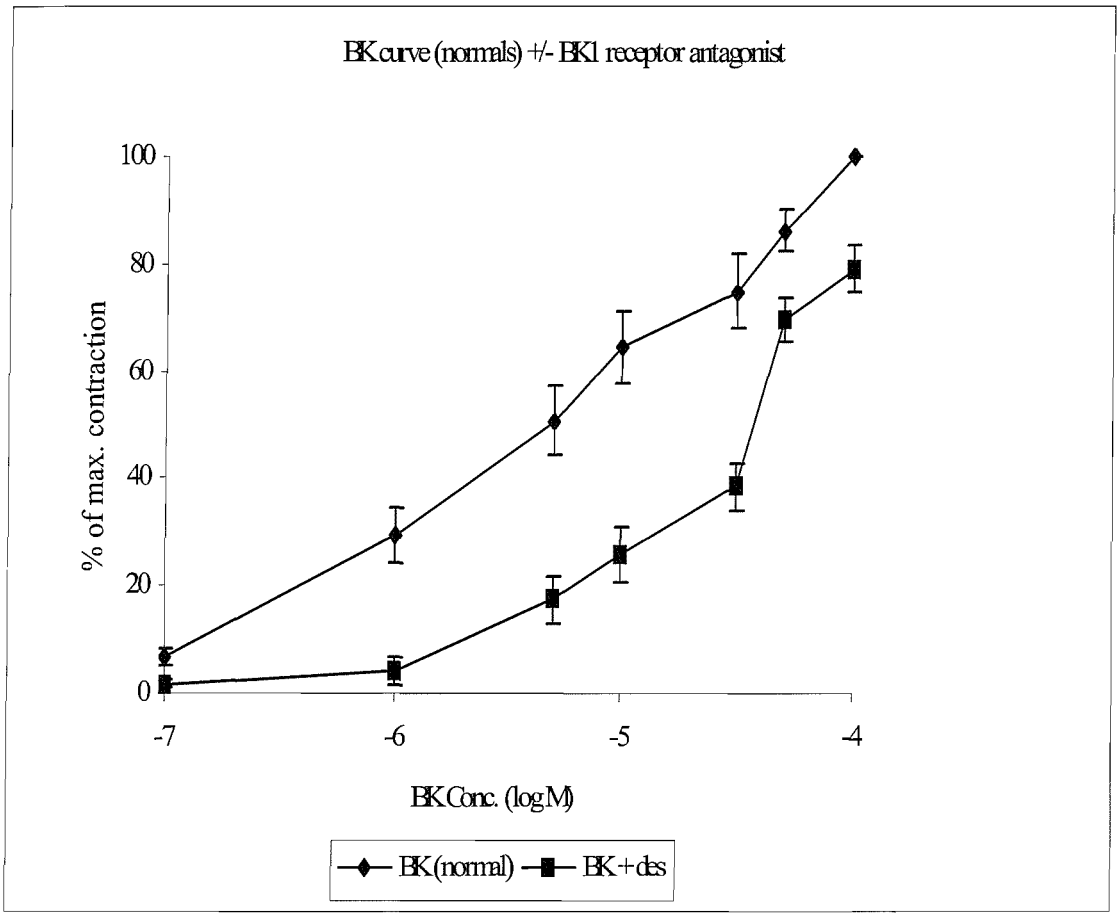


Fig 4.3 The effect of the BK_1 receptor antagonist des-Arg^9-[Leu^8]-BK on BK induced contraction of normal human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E of 6 experiments. BK concentration response curves (CRCs) were constructed in the absence or in the presence of des-Arg^9-[Leu^8]-BK (1μM). $_pKB$= 6.5±0.02. Data are expressed as a percentage of the maximum contraction obtained from the first response curve to BK. $_pKB$ (-log K_B) was obtained according to the equation: K_B = ([A]/concentration ratio-1), where [A] is the concentration of the antagonist and the concentration ratio is the ED_{50} in the presence of the antagonist divided by the ED_{50} in the absence of the antagonist.

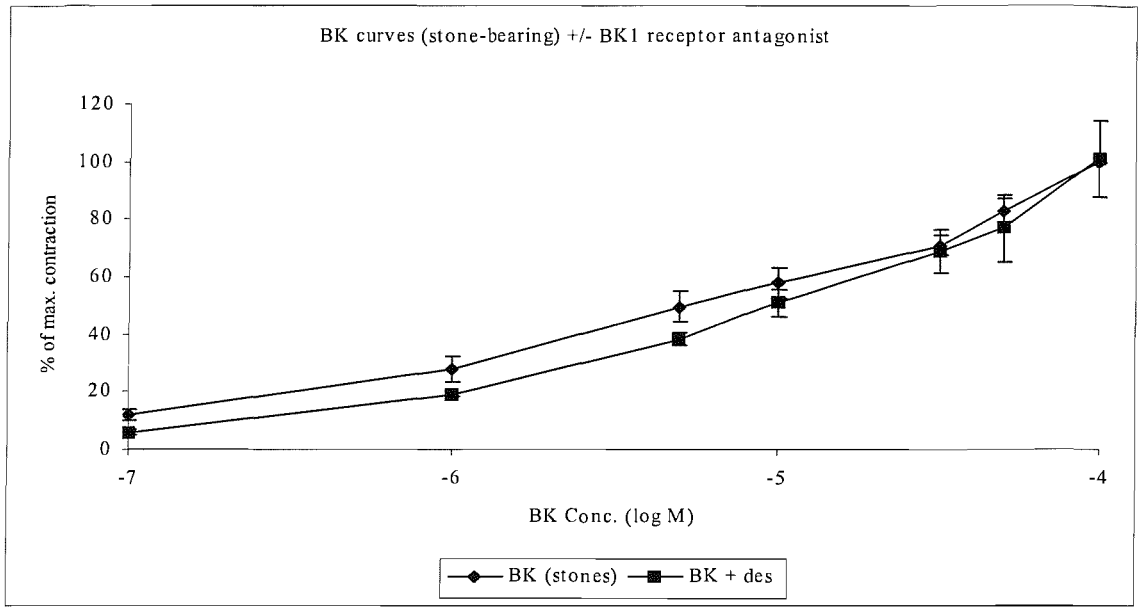


Fig 4.4 The effect of the BK_1 receptor antagonist des-Arg^9-[Leu^8]-BK on BK induced contraction of stone-bearing human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E of 9 experiments. BK CRCs were constructed in the absence or in the presence of antagonist des-Arg^9-[Leu^8]-BK (1μM). $_PKB$= 5.83±0.18. The data expressed above and the $_PKB$ values were calculated as stated in figure 4.3.

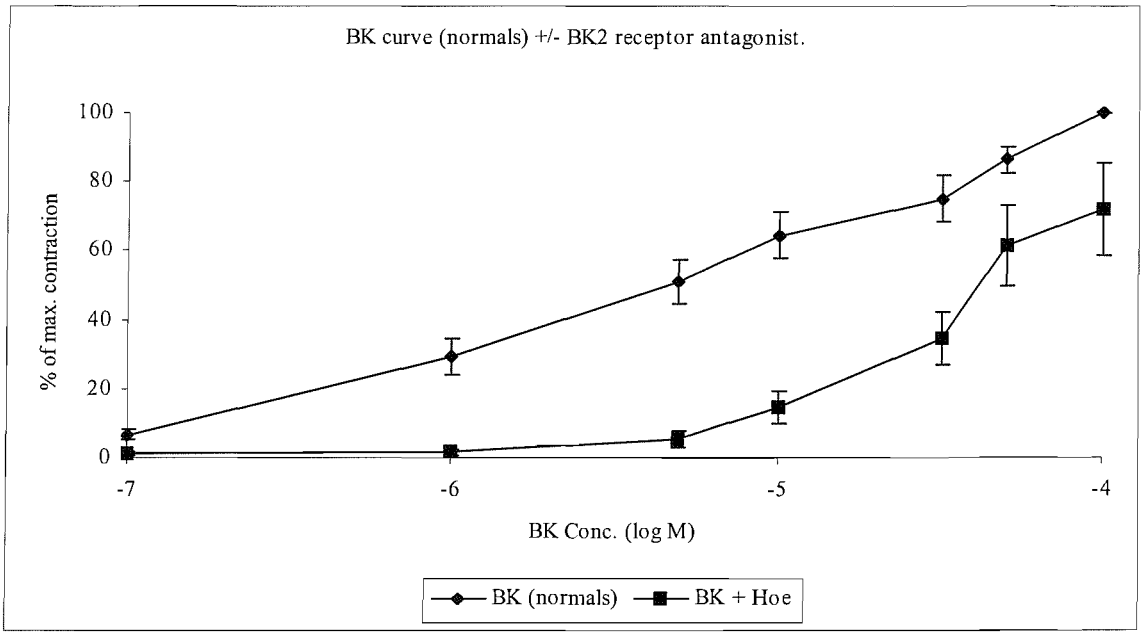


Fig 4.5 The effect of the BK_2 receptor antagonist HOE-140 on BK induced contraction of normal human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E of 6 experiments. BK CRCs were constructed in the absence or in the presence of HOE-140 (1μM). $_PKB$= 6.53±0.12. The data expressed above and the $_PKB$ values were calculated as stated in fig 4.3.

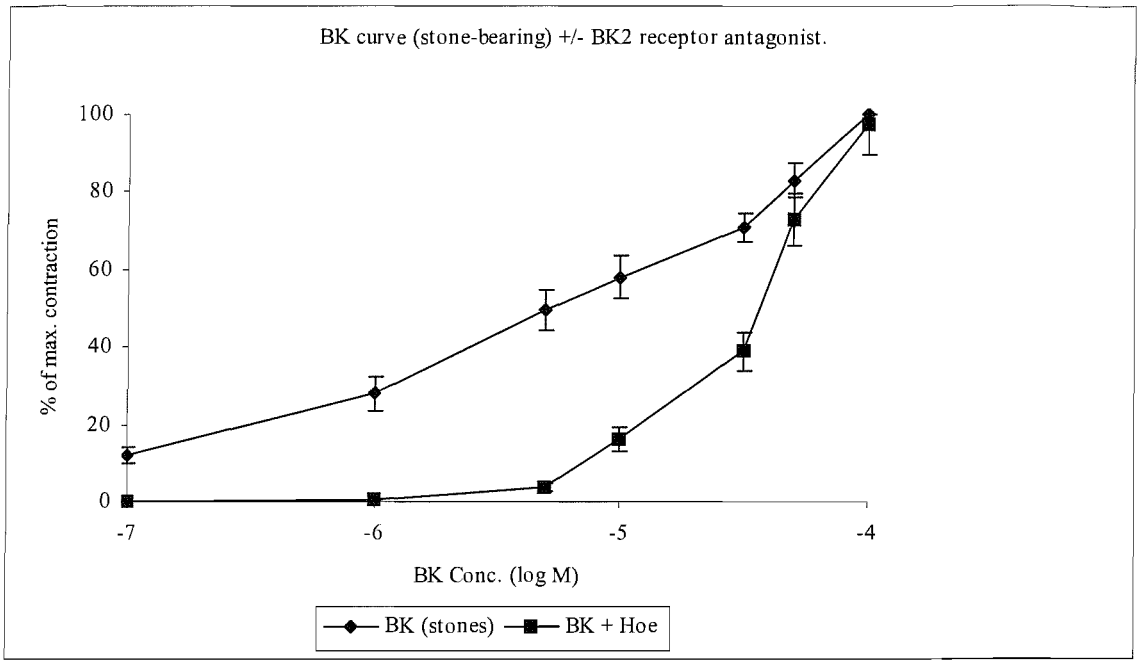


Fig 4.6 The effect of the BK_2 receptor antagonist HOE-140 on BK induced contraction of stone-bearing human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E of 5 experiments. BK CRCs were constructed in the absence or in the presence of HOE-140 (1μM). $_pKB = 6.6 \pm 0.07$. The data expressed above and the $_pKB$ values were calculated as stated in fig. 4.3.

Each of the responses above was obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM).

4.1.4 BK1 and BK2 receptors combined

The response of combined BK1 and BK2 blockade was investigated to see if there was any evidence of an additive blockade effect in normal or stone-bearing strips. When both the BK1

and BK2 receptors were blocked simultaneously there was no significant additive effect in normal or stone-bearing gallbladder strips (fig 4.7 & 4.8).

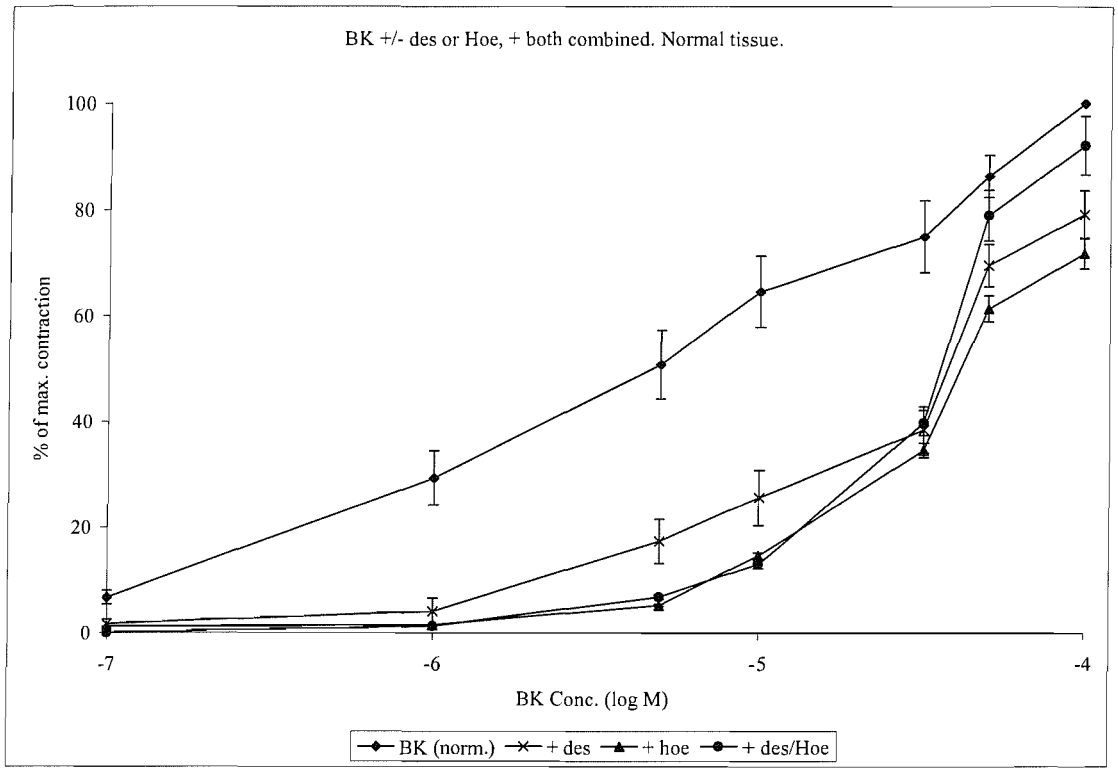


Fig 4.7 This figure shows the effect of the BK receptors (individually and in combination) on BK induced contraction of normal human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E of 4-6 experiments. BK concentration response curves (CRCs) were constructed in the absence or in the presence of des-Arg^9-[Leu^8]-BK (1μM), Hoe-140 (1μM) or in the presence of 1μM of both receptor antagonists combined. Data are expressed as a percentage of the maximum contraction obtained from the first response curve to BK. No additional level of blockade was achieved with both antagonists in normal tissue.

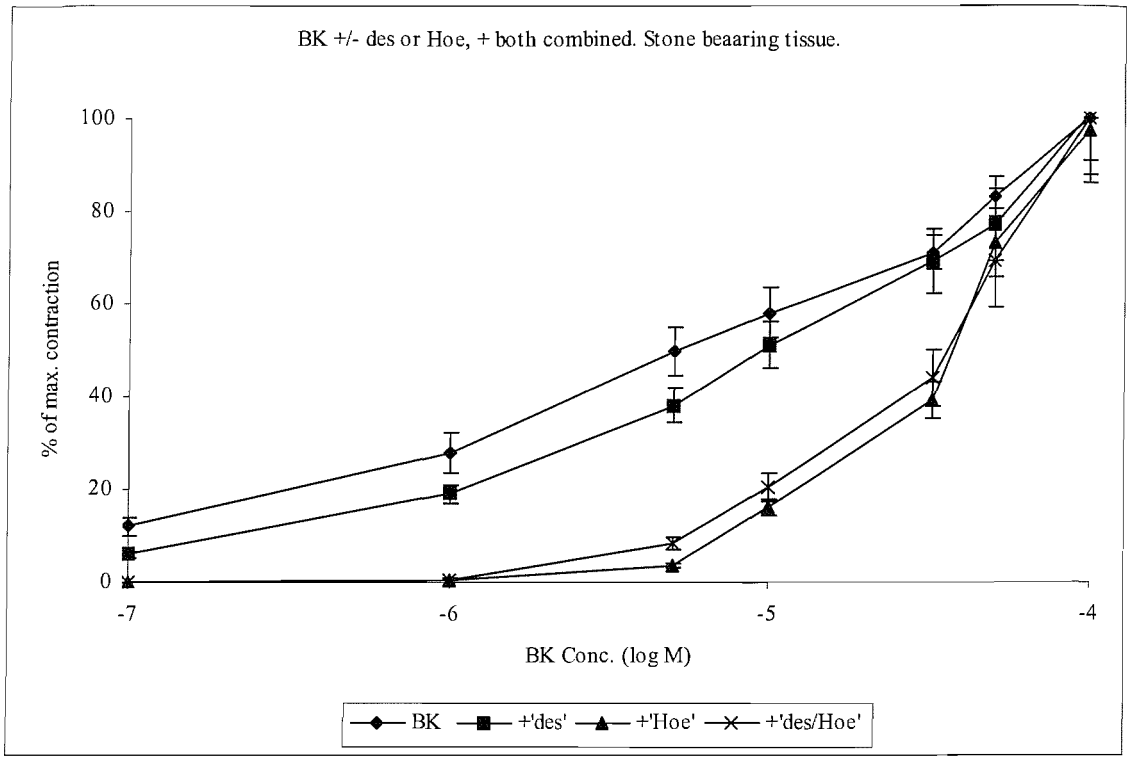


Fig 4.8 This figure shows the effect of the BK receptors (individually and in combination) on BK induced contraction of stone-bearing human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E of 4-6 experiments. BK concentration response curves (CRCs) were constructed in the absence or in the presence of des-Arg^9-[Leu^8]-BK (1μM), Hoe-140 (1μM) or in the presence of 1μM of both receptor antagonists combined. Data are expressed as a percentage of the maximum contraction obtained from the first response curve to BK. No additional level of blockade was achieved with both antagonists in stone-bearing tissue.

4.1.5 Bradykinin 1 receptor agonist

As previously mentioned the results, so far, suggest that both BK1 and BK2 receptors exist in both normal and stone-bearing gallbladder tissue. The results also indicate that a difference in BK receptor population exists between the two types of tissue. Whether this is due to an up-regulation of the BK2 receptors or a down regulation of the BK1 receptors, in stone-bearing tissue is unclear. BK1 receptor seems to be more 'involved' in BK contraction in normal tissue. To examine this theory a concentration response curve was obtained for a BK1 receptor

agonist (des-Arg^9-BK) in both normal and stone-bearing gallbladders. The normal strips showed greater contraction in response to the BK1 receptor agonist at 50% contraction of the maximum (ED_{50} = 2.5&1.0 nM). When the BK1 receptor antagonist was present, the only significant rightward shift of the curve was in normal tissue. ED_{50}= 5.3 (stone-bearing), 50% of the maximum contraction, was not reached in normal tissue. (fig 4.9).

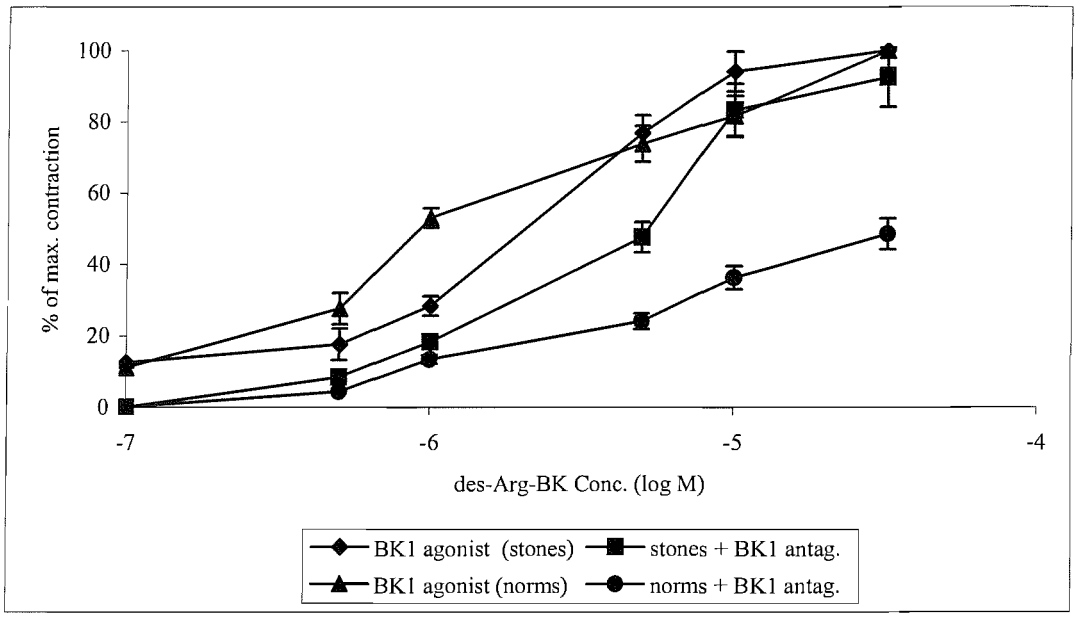


Fig 4.9 The effect of the BK_1 receptor antagonist des-Arg^9-[Leu^8]-BK on des-Arg^9-BK (a BK_1 receptor agonist) induced contraction of normal and stone-bearing human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of des-Arg^9-BK (0.1-20μM). Each point represents the mean ± S.E of 4 experiments. des-Arg^9-BK CRCs were constructed in the absence or in the presence of antagonist des-Arg^9-[Leu^8]-BK (1μM). The data expressed above was calculated as stated in figure 4.3.

4.1.6 Maximum BK response

It is worth noting, at this stage, that despite some large and significant rightward shifts of the curves the maximum BK response neither antagonist (1μM) was blocking the maximum BK

response (fig 4.2-4.8) and the antagonist's affect was less apparent. An explanation for this is that the high BK concentration overcomes the competitive inhibition, so to test this theory, BK response curves were obtained with different concentrations of each BK antagonist. Three different concentrations of both BK receptor antagonists were used. In addition to the experiments described above with concentrations of 1μM, the BK concentration curve was repeated in the presence of des-Arg^{9}-Leu^{8}- BK (100,500nM) and HOE-140 (30,100nM) (figs 4.10 & 4.11). From the figures below one can see that a change in the concentration of the receptor antagonists had the same effect on the slope. Therefore increasing the concentration would still not affect the maximum contraction.

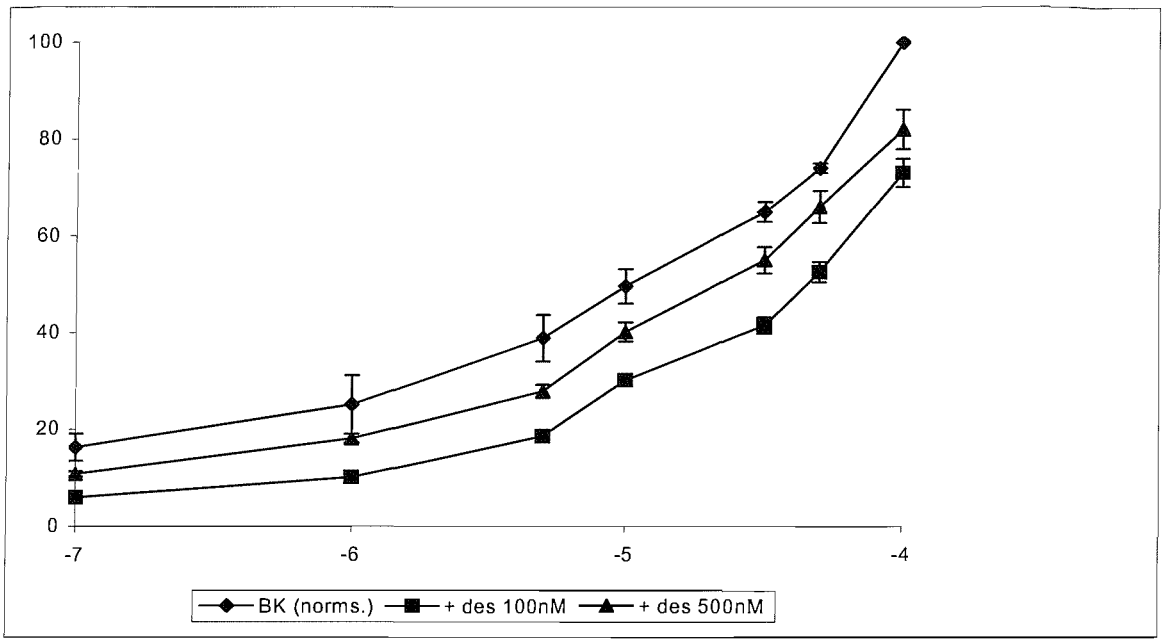


Fig 4.10 (a) A comparison of two different concentrations of the BK_1 receptor antagonist, des-Arg^9-[Leu^8]-BK on normal human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM).. Each point represents the mean ± S.E. mean of 5 experiments. BK concentration response curves (CRCs) were constructed in the absence of the receptor antagonist and then in the presence of des-Arg^9-[Leu^8]-BK (100nM) and then des-Arg^9-[Leu^8]-BK (500nM). Data expressed as stated in figure 4.3.

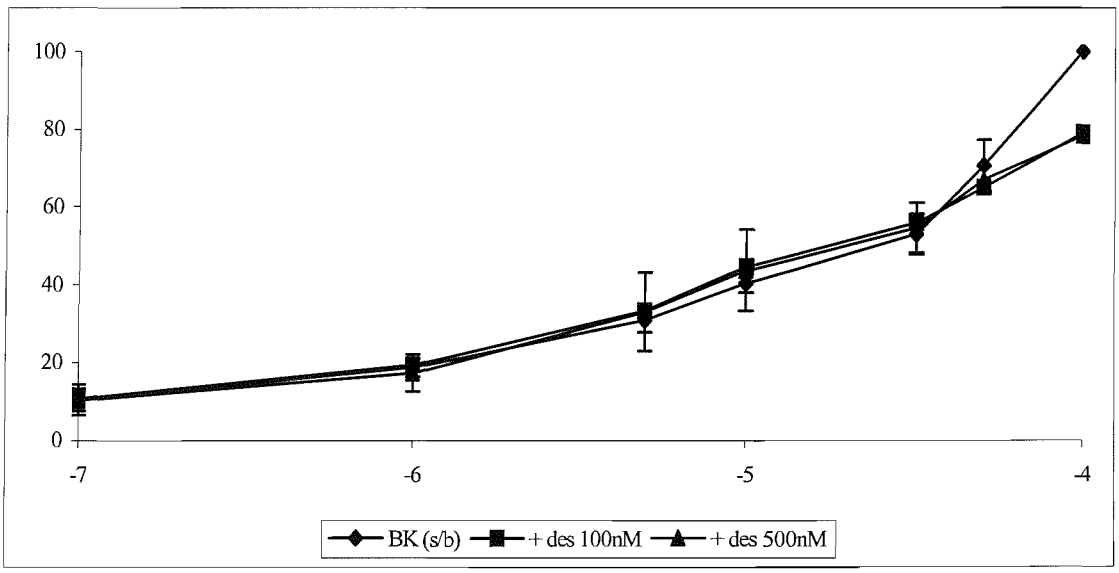


Fig 4.10 (b) A comparison of two different concentrations of the BK_1 receptor antagonist, des-Arg^9-[Leu^8]-BK on stone-bearing gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK BK (0.1-100μM)... Each point represents the mean ± S.E. mean of 5 experiments. BK concentration response curves (CRCs) were constructed in the absence of the receptor antagonist and then in the presence of des-Arg^9-[Leu^8]-BK (100nM) and then des-Arg^9-[Leu^8]-BK (500nM). Data expressed as stated in figure 4.3.

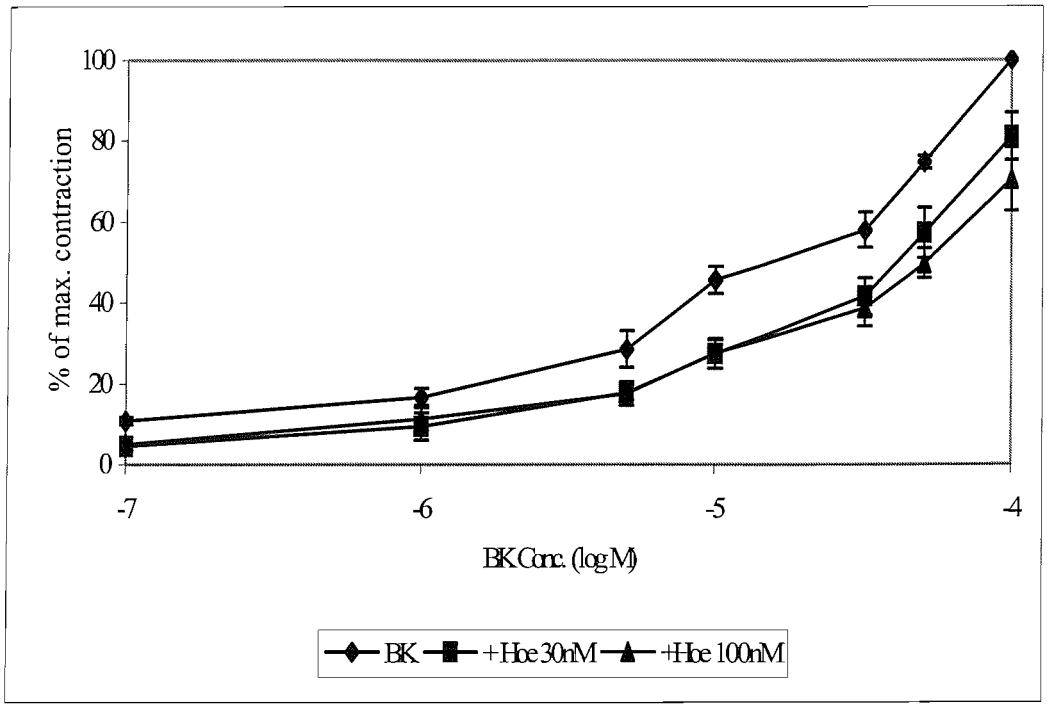


Fig 4.11 A comparison of two different concentrations of the BK2 receptor antagonist, Hoe-140 on human gallbladder (normal and stone-bearing combined) tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E. mean of 5 experiments. BK concentration response curves (CRCs) were constructed in the absence of the receptor antagonist and then in the presence of Hoe-140 (30nM) and then Hoe-140 (100nM). Data expressed as stated in figure 4.3

4.1.7 Vilability testing

To confirm the viability of the strips and the repeatability of the BK curve at the end of the run of experiments, strips that were still deemed viable were subjected to a final BK concentration response curve. Viable strips were repeatedly washed out and allowed to return to a stable baseline and after 30 minutes of equilibration they were subjected to a repeat BK concentration curve. The repeat BK curve showed a significant leftward shift when compared to the BK curve with the antagonists present and it returned towards the original shape, ANOVA P=0.04, F=6.43

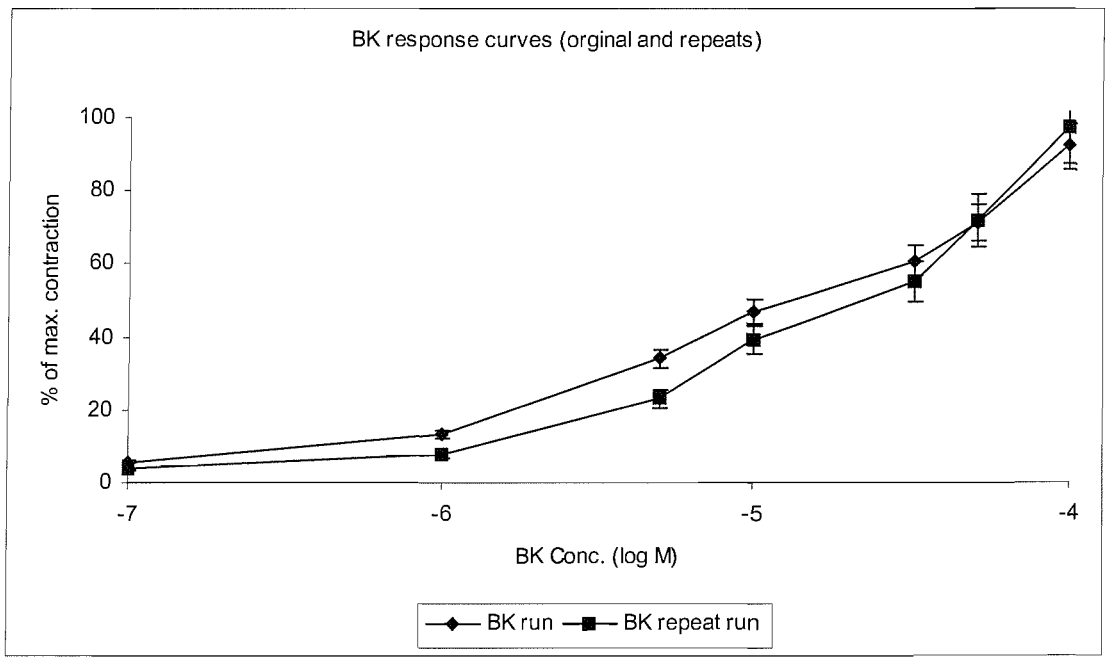


Fig 4.12 In a number of initial experiments BK curves were repeated after all antagonists were washed out and when a stable baseline had returned. The initial BK run was achieved after two hours equilibration, the various antagonists were then added, as described previously (figs 4.3-4.6), then after a number of washouts and when a stable baseline had returned a repeat BK curve was obtained. Each strip was expose to cumulative increases of BK (0.1-100μM) and each point represents the mean of 5 experiments ± S.E over a typical 6 hour experiment.

4.2 Independent mechanisms of BK contraction

The first part of the results section (4.1) used BK agonists and antagonists as pharmacological tools to investigate the role of BK on normal and stone-bearing gallbladder tissue. The experiments have attempted to establish that: both types of tissue contract in a dose-dependent manner to BK and that both BK1 and BK2 receptors mediate this action. A difference in BK receptor population seems to exist between normal and stone-bearing tissues. The possibility of a third BK receptor or the possibility that BK may act through another independent mechanism is the focus of the next part of the result section deals with this possibility.

4.2.1 Cholecystokinin (CCK)

CCK is widely distributed throughout the gastrointestinal tract. It stimulates gallbladder contractions and intestinal motility. The factors that control the release of CCK include neural and hormonal mechanisms and intestinal luminal factors. CCK is the main hormone that is involved in gallbladder motility and it acts on the gallbladder muscle cells directly (Schjoldager et al 1989). As CCK is of such importance to gallbladder motility – any link with the BK receptor mechanisms was examined.

Initially, like for bradykinin, a dose response curve for CCK needed to be established. This was achieved in a similar fashion to the BK response curve i.e. CCK was incrementally added (0.1–10μM). The table below shows that only at the concentration of 10^{-8}, did all strips contract to at least 1% of maximum. The mean of all six strips at 10^{-9} only showed a contract

of just 3% of the maximum. A contraction within 2% of the recorded maximum was reached in all strips at 10^{-6} and the mean of all strips showed a contraction of 99% of the maximum. Therefore, all subsequent CCK dose response curves were obtained using the range 10nM-1μM.

CCK (M)	10^{-9}	10^{-8}	$2x10^{-8}$	$5x10^{-8}$	10^{-7}	$2x10^{-7}$	$5x10^{-7}$	10^{-6}	10^{-5}	10^{-4}
B1	0	7	14.4	25	29.8	59	63	100	100	99.8
B2	0.4	9	19	26.2	34.6	40	74	98	99	100
B3	7	14	20	35	48	53	76.8	99.4	99.6	100
B4	4	26	32	50	65.9	74	83	99	99.7	100
B5	6	14	20	24	61	76	94	100	99.4	99.1
B6	0.6	4	9	15	29	50	81	98	100	99.0
Mean	**3**	**12.3**	**19**	**29.2**	**44.7**	**58.7**	**78.6**	**99**	**99.6**	**99.65**

Table 4.3 Contraction of human gallbladder strips *in vitro* in response to Cholecystokinin in the range 1n-$1x10^{-4}$M, expressed as a % of maximum the contraction. The six strips are from three different gallbladders.

4.2.1.1 CCK dose response curves and BK receptor interaction

Both normal and stone-bearing gallbladder strips were subjected to the CCK contraction response curve. As figure 4.14 shows both normal and stone-bearing gallbladder strips similar and repeatable response curves to CCK (10nM-1μM). ED_{50}= 77.8 and 111.2nM respectively.

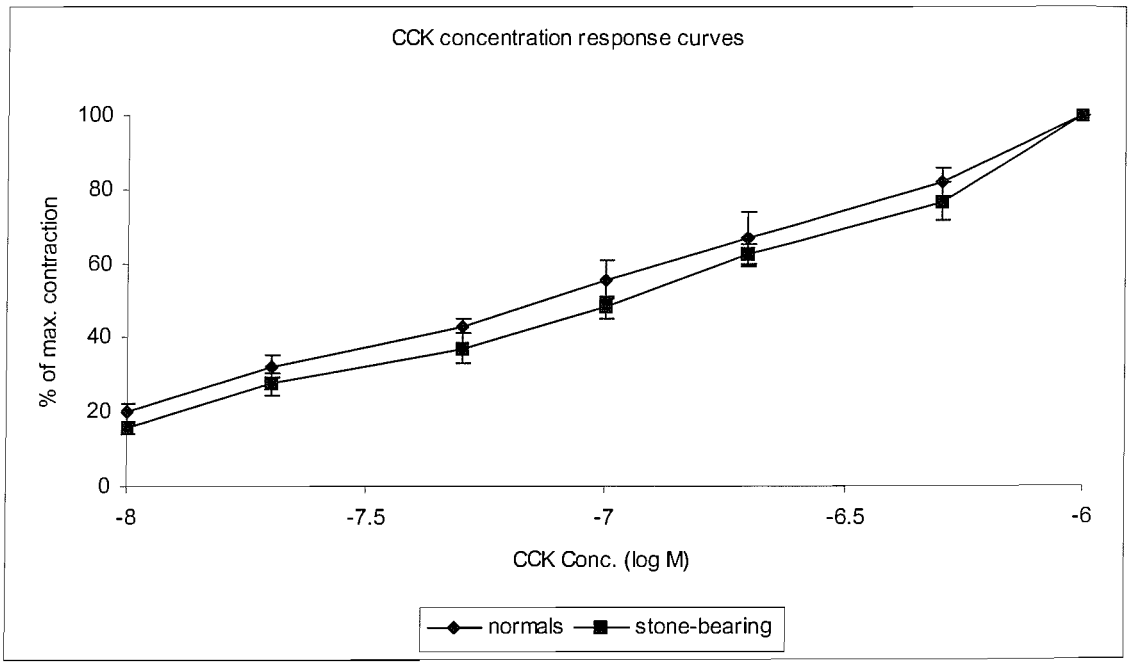


Fig 4.14 Cholecystokinin concentration response curves for normal and stone-bearing gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of CCK (1-1000nM). Each point represents the mean ± S.E. mean of 5-6 experiments. The ED_{50} values obtained for normal and stone-bearing are 77.8nM and 111.2nM respectively.

It was decided to try and see if the CCK response might be mediated through the BK receptor mechanism. The CCK response curves were obtained for both normal and stone-bearing tissue and then they were repeated in the presence of each BK receptor antagonist i.e. des-Arg^9-[Leu^8]-BK (1μM), Hoe-140 (1μM) and also with both receptor antagonists combined. When the BK1 receptor antagonist des-Arg^9-[Leu^8]-BK (1μM) was introduced a significant rightward shift of the curve was achieved in normal tissue only; ANOVA P=0.002, F=7.79.

ED_{50} = 77.8nM (absence), 287nM (presence). The addition of the BK2 receptor antagonist, Hoe-140 (1μM) resulted in a significant rightward of the curve in both normal and stone-bearing gallbladder strips; ANOVA P=0.001 & 0.002, F=13.12 & 10.54 respectively. Normal strips : ED_{50} = 77.8nM (absence of antagonist), 863nM (presence of antagonist). Stone-bearing strips : ED_{50} = 111.2 (absence), 360nM (presence). Significant additional level of blockade was noted in the presence of both BK receptor antagonists combined, for both normal and stone-bearing gallbladder strips. ED_{50} = 835nM (stone-bearing). 50% of the maximum contraction was not reached in normal issue.

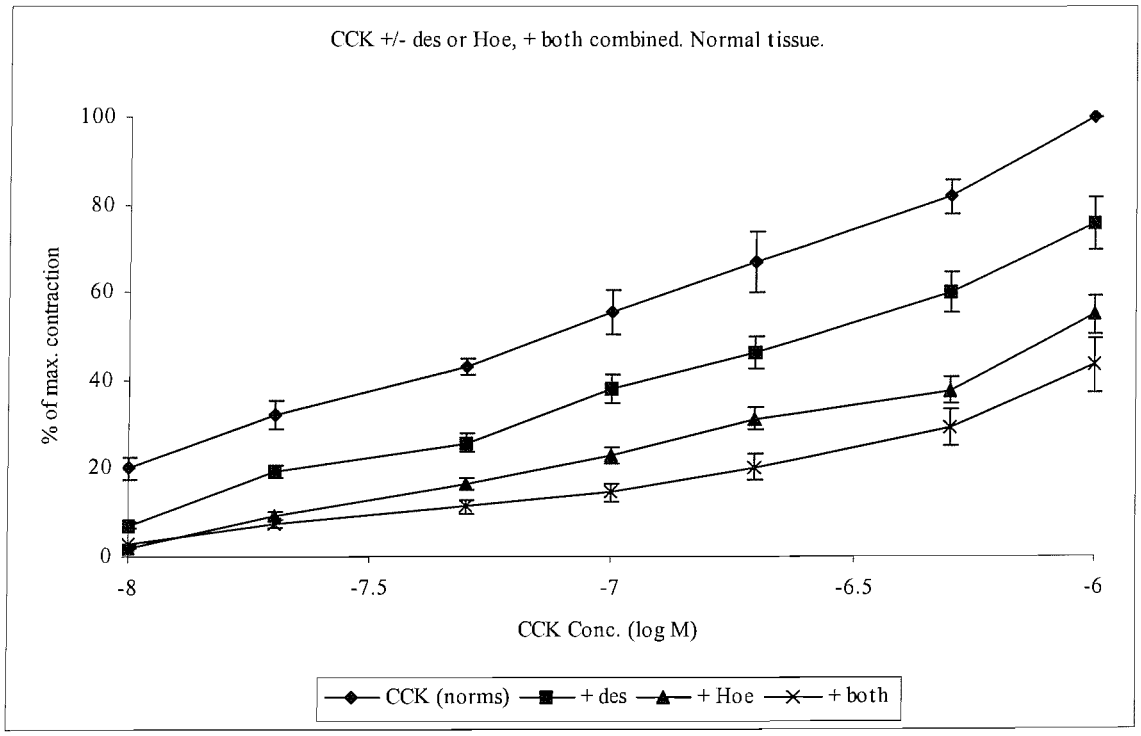


Fig 4.15 The effect of the BK_1 receptor antagonist, the BK_2 receptor antagonist and both receptor antagonists combined on CCK induced contraction of normal human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of CCK (10nM-1μM). Each point represents the mean ± S.E of 6 experiments. CCK concentration response curves (CRCs) were constructed in the absence or in the presence of des-Arg^9-[Leu^8]-BK (1μM), Hoe-140 (1μM) or both receptor antagonists combined. Data are expressed as a percentage of the maximum contraction obtained from the first response curve to CCK.

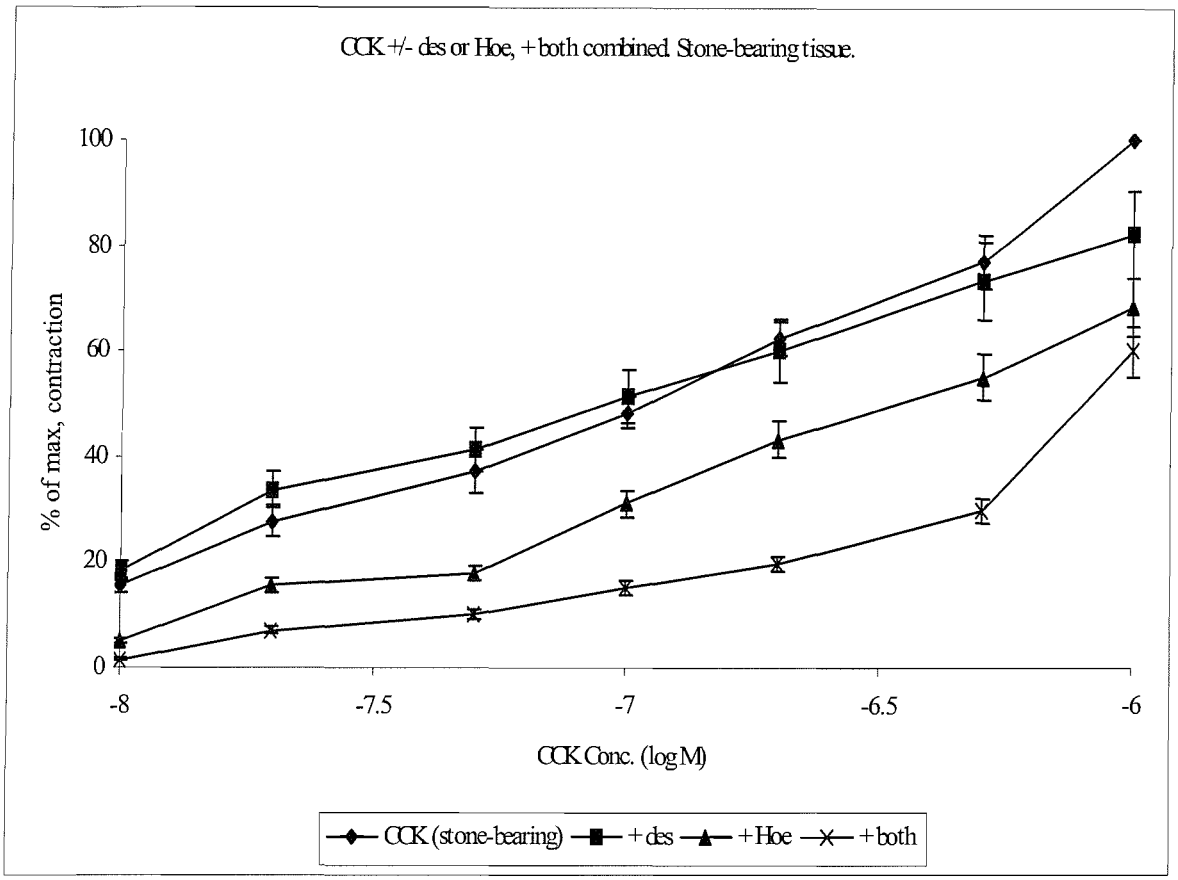


Fig 4.16 The effect of the BK_1 receptor antagonist, the BK_2 receptor antagonist and both receptor antagonists combined on CCK induced contraction of stone-bearing human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of CCK (1nM-1μM). Each point represents the mean ± S.E. mean of 6 experiments. CCK concentration response curves (CRCs) were constructed in the absence or in the presence of des-Arg^9-[Leu^8]-BK (1μM), Hoe-140 (1μM) or both receptor antagonists combined.

4.2.1.2 CCK receptor

As discussed previously, the receptor involved in CCK induced gallbladder motility is the CCK-A receptor. A known CCK-A receptor antagonist, (SR27897) was introduced. The initial aim was to establish whether SR27897 actually blocked the CCK receptors involved in CCK gallbladder contraction (as noted in figure 4.14, above). A CCK dose response was achieved and the then SR27897 (0.5nM) was added and the curve repeated.

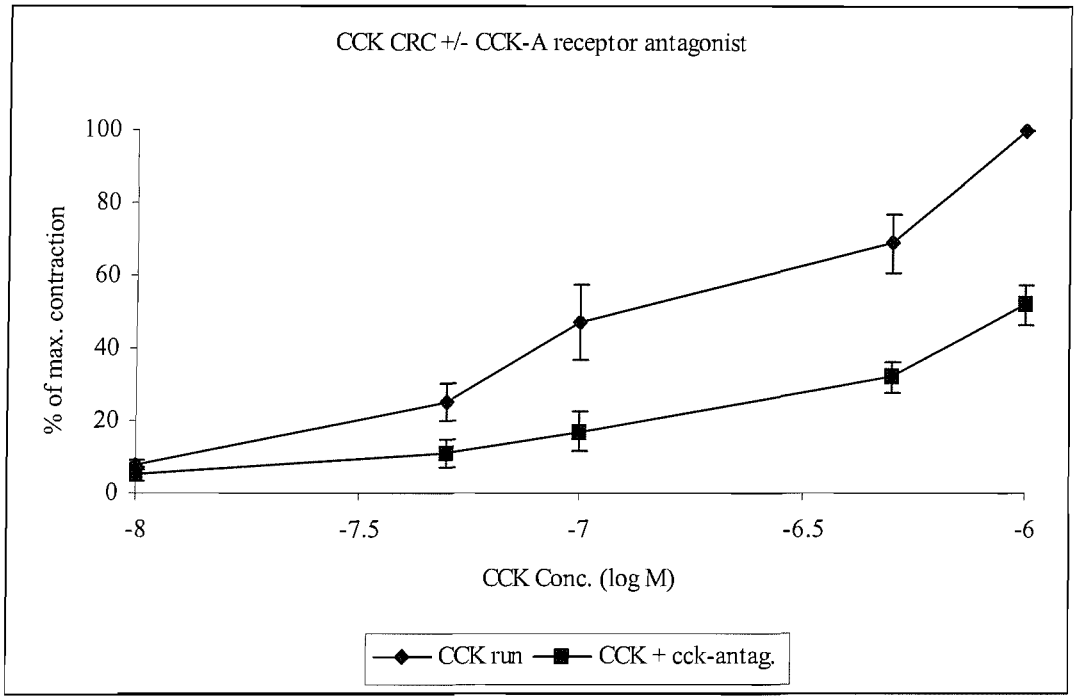


Fig 4.17 The effect of the CCK-A receptor antagonist, SR27897, on CCK induced contraction of human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of CCK (10-1000nM). Each point represents the mean ± S.E of 6 experiments. CCK concentration response curves (CRCs) were constructed in the absence or in the presence of SR27897 (0.5nM). $_{P}KB= 6.5\pm0.02$. Values were calculated as previously described.

4.2.1.3 Possible BK and CCK receptor interaction

The next step was to establish whether BK action was mediated by the CCK-A receptor. In the next two figures (fig 4.18-4.19) a BK dose response was obtained for normal and stone-bearing gallbladder strips and both curves were repeated in the presence of the CCK-A receptor antagonist (SR27897 (0.5nM)). No significant rightward shifts of the curve were achieved in either case. Therefore, the CCK receptor did not mediate the BK response.

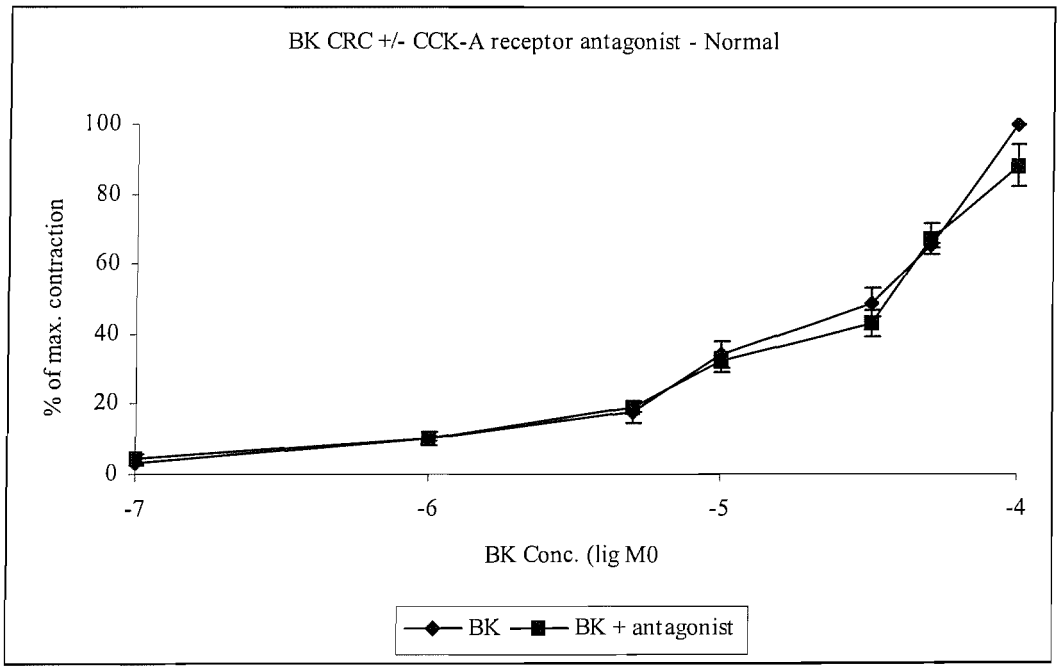


Fig 4.18 The effect of the CCK-A receptor antagonist, SR27897, on BK induced contraction of normal human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (1-100μM). Each point represents the mean ± S.E of 3 experiments. BK concentration response curves (CRCs) were constructed in the absence or in the presence of SR27897 (0.5nM). No significant shift of the curve was noted.

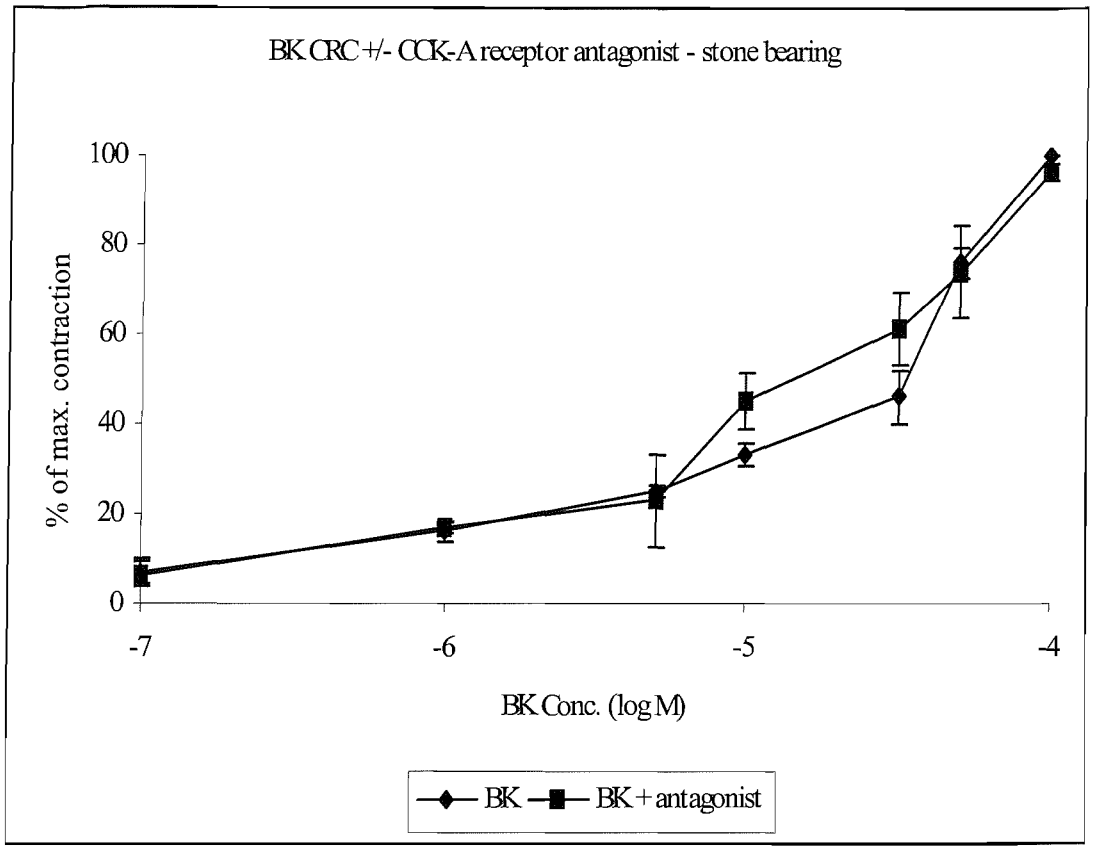


Fig 4.19 The effect of the CCK-A receptor antagonist, SR27897, on BK induced contraction of stone-bearing gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (1-100μM). Each point represents the mean ± S.E of 3 experiments. BK concentration response curves (CRCs) were constructed in the absence or in the presence of SR27897 (0.5nM). No significant shift of the curve was noted.

4.2.2 Various other possible receptors, channel mediators

As previously discussed in section two – the interaction of BK with other mechanisms needed to be explored – sodium, potassium, calcium channels, NANC response etc. As the various receptors and channels have already been discussed in detail, only a brief introduction is given for each channel, receptor blocker/antagonist. With each experiment performed, if no significant rightward shift of the curve was noted the results for normal and stone-bearing gallbladder strips were not separated into different figures.

Histamine is an endogenous compound that is synthesized, stored and released primarily by mast cells and after release exerts its effect on many tissues and organs. The actions of histamine are mediated through at least 3 distinct receptors H1, H2 and H3. The receptors involved in the gastrointestinal tract are H1 and H2. Meyramine blocks both of these receptors. A BK response curve (0.1-100μM) was established and was then repeated in the presence of mepyramine (1μM) see- figure 4.20. No significant rightward shift of the curve was noted (ANOVA P=0.08, F=1.16) – therefore the histamine receptors do not mediate the BK action.

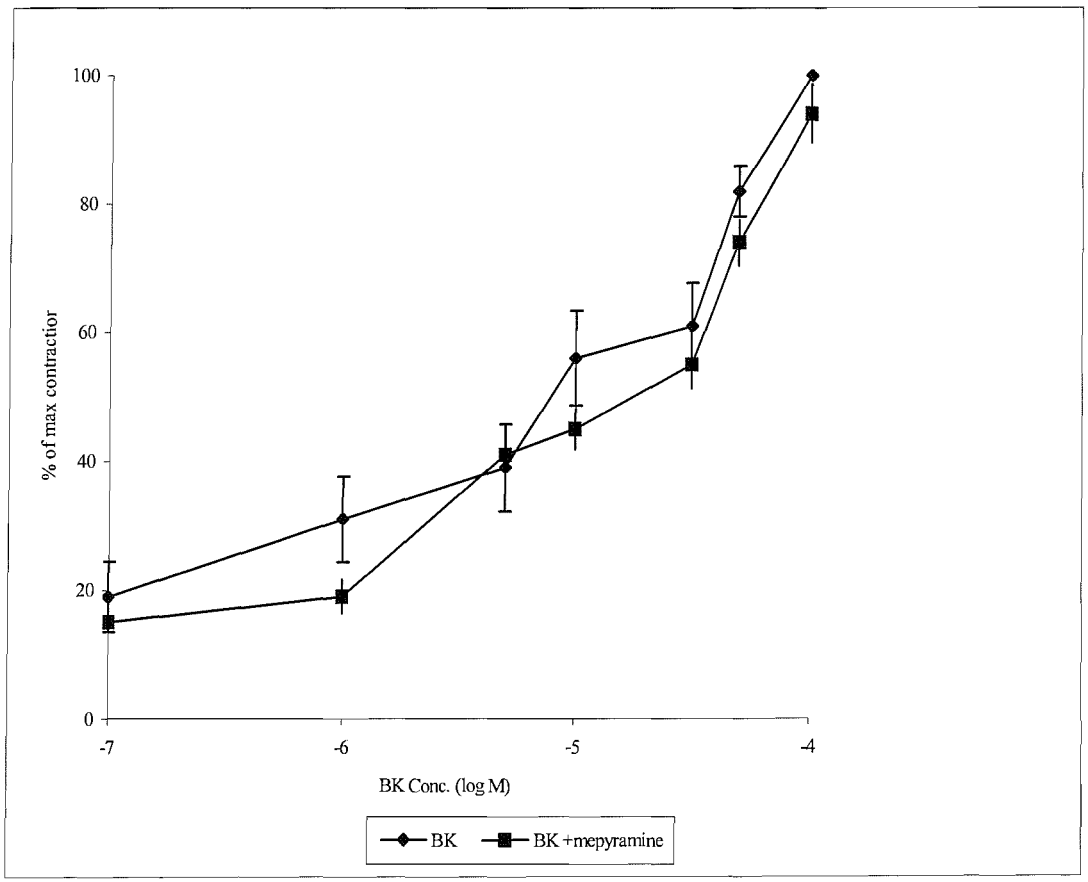


Fig 4.20. The effect of a histamine receptor antagonist on BK induced contraction of human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E. mean of 4 experiments. BK concentration response curves (CRCs) were constructed in the absence or in the presence of mepyramine (1μM). $_{P}$KB= 5.67. Values were calculated as previously described.

Cholinergic receptors can be sub-classed into nicotinic or muscarinic receptors. **Muscarinic** receptors may also be sub-divided into at least two subtypes – M1 and M2, based on the pharmacological specificities of certain agonists and antagonists. Atropine blocks both M1 and M2 muscarinic cholinergic receptors to nearly equivalent extent. A BK response curve (0.1-100μM) was established and was then repeated in the presence of atropine (1μM)- see figure 4.21. No significant rightward shift (ANOVA P=0.831, F=0.046) of the curve was noted therefore the BK response was non-muscarinic.

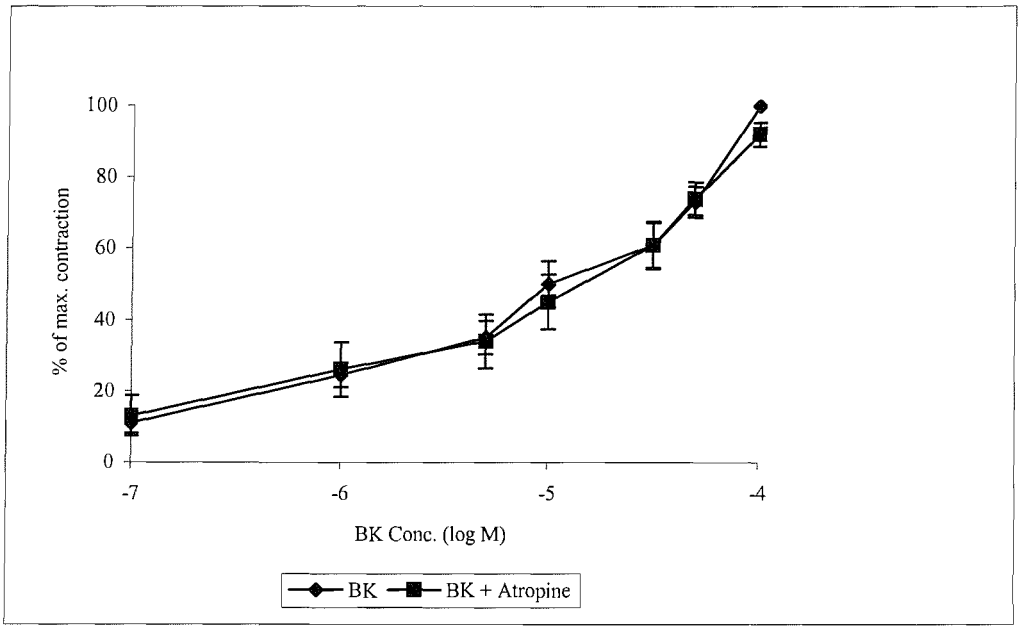


Fig 4.21 The effect of a muscarinic receptor antagonist on BK induced contraction of human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E. mean of 7 experiments. BK concentration response curves (CRCs) were constructed in the absence or in the presence of atropine (1μM). $_{P}$KB= 4.91. Values were calculated as previously described.

The **adrenergic response** involved in gallbladder smooth muscle motility, as previously discussed, is mediated through the α1- adrenergic receptors. Prazosin blocks the α1-adrenergic receptor. A BK response curve (0.1-100μM) was established and was then repeated in the presence (1μM) – figure 4.22. No significant rightward shift of the curve was noted (ANOVA P=0.296, F=1.103) – therefore the BK response is non-adrenergic.

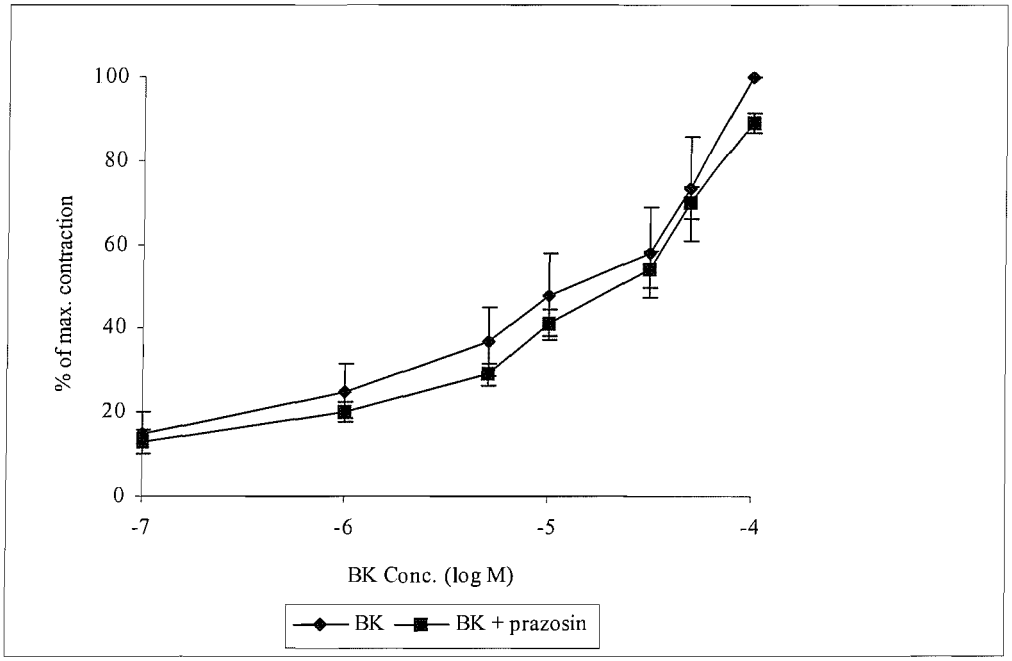


Fig 4.22 The effect of a α_1-adrenoceptor antagonist on BK induced contraction of human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E. mean of 6 experiments. BK concentration response curves (CRCs) were constructed in the absence or in the presence of prazosin (0.1μM).

The next possible mediator of the BK response was to look at the **sodium and potassium channels**. They both were previously discussed in detail and in conjunction with any possible interaction with BK. Many antagonists block voltage dependent Na channels and thereby make it more difficult, or impossible, for an action potential to be produced. Tetrodotoxin (TTX) is one of the most potent agents that is known for blocking these channels. A BK response curve (0.1-100μM) was established and was then repeated in the presence of TTX (0.3μM) – figure 4.23. Quinidine blocks the potassium channels. A BK response curve (0.1-100μM) was established and was then repeated in the presence of quinidine (1μM) – figure 4.24. No significant rightward shift of either curve was noted (ANOVA P=0.57 & 0.188, F=0.325 & 1.755 respectively) – therefore the BK response is not mediated through the Na/K channels.

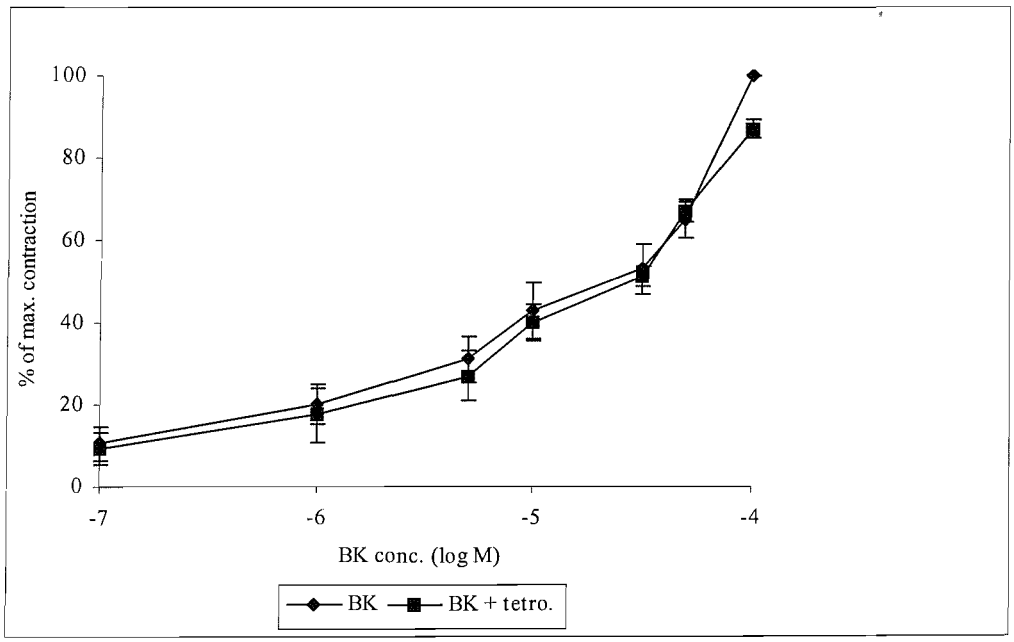


Fig 4.23 The effect of a Na^+ channel blocker on BK induced contraction of human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E. mean of 5 experiments. BK concentration response curves (CRCs) were constructed in the absence or in the presence of tetrodotoxin (0.3μM).

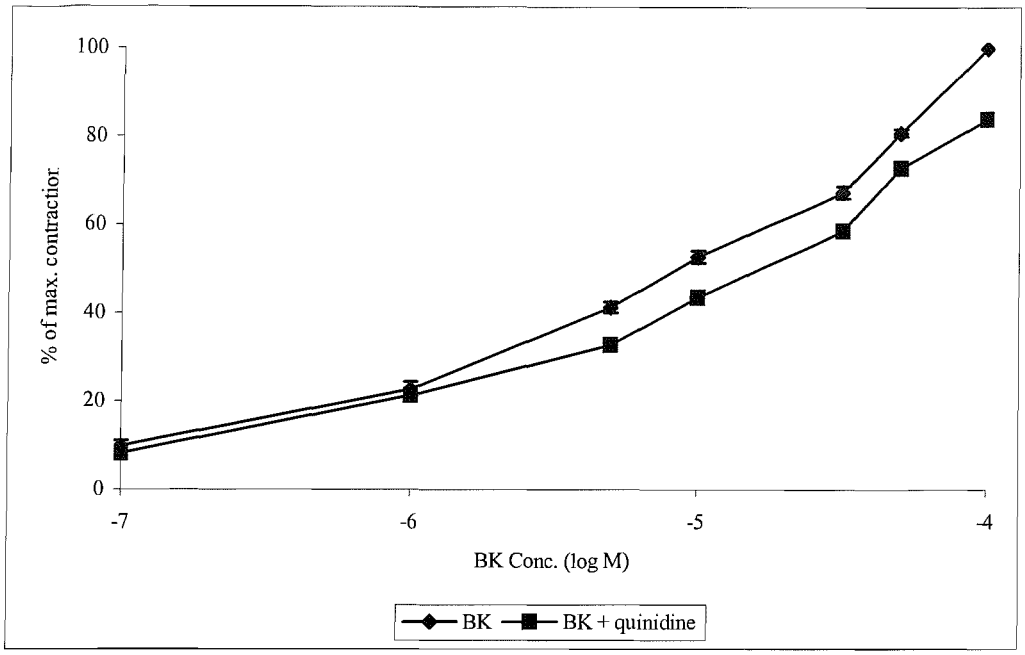


Fig 4.24 The effect of a K^+-channel blocker on BK induced contraction of human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E. mean of 7 experiments. BK concentration response curves (CRCs) were constructed in the absence or in the presence of quinidine (1μM). $_pKB$= 4.75. The data expressed above and the $_pKB$ values were calculated as previously stated.

The involvement of both **intracellular and extracellular calcium** needed to be examined. The importance of both was previously discussed. Ryanodine is a neutral alkaloid that affects the opening and closing of calcium channels. A BK response curve (0.1-100μM) was established and was then repeated in the presence of ryanodine (10μM)- see figure 4.25. A significant rightward shift (ANOVA P=0.003, F=9.441) of the entire curve was noted therefore the BK response requires the presence of intracellular calcium. The involvement of extracellular Ca^+ on BK was then examined. . A BK response curve (0.1-100μM) was established for each strip in Krebs solution containing calcium and in calcium free Krebs solution figure 4.26. The calcium free krebs solution contained EGTA. No significant rightward shift of the curve was noted (ANOVA P=0.904, F=0.15) therefore the BK response is extracellular calcium independent but as the previous experiment shows intracellular calcium is vital.

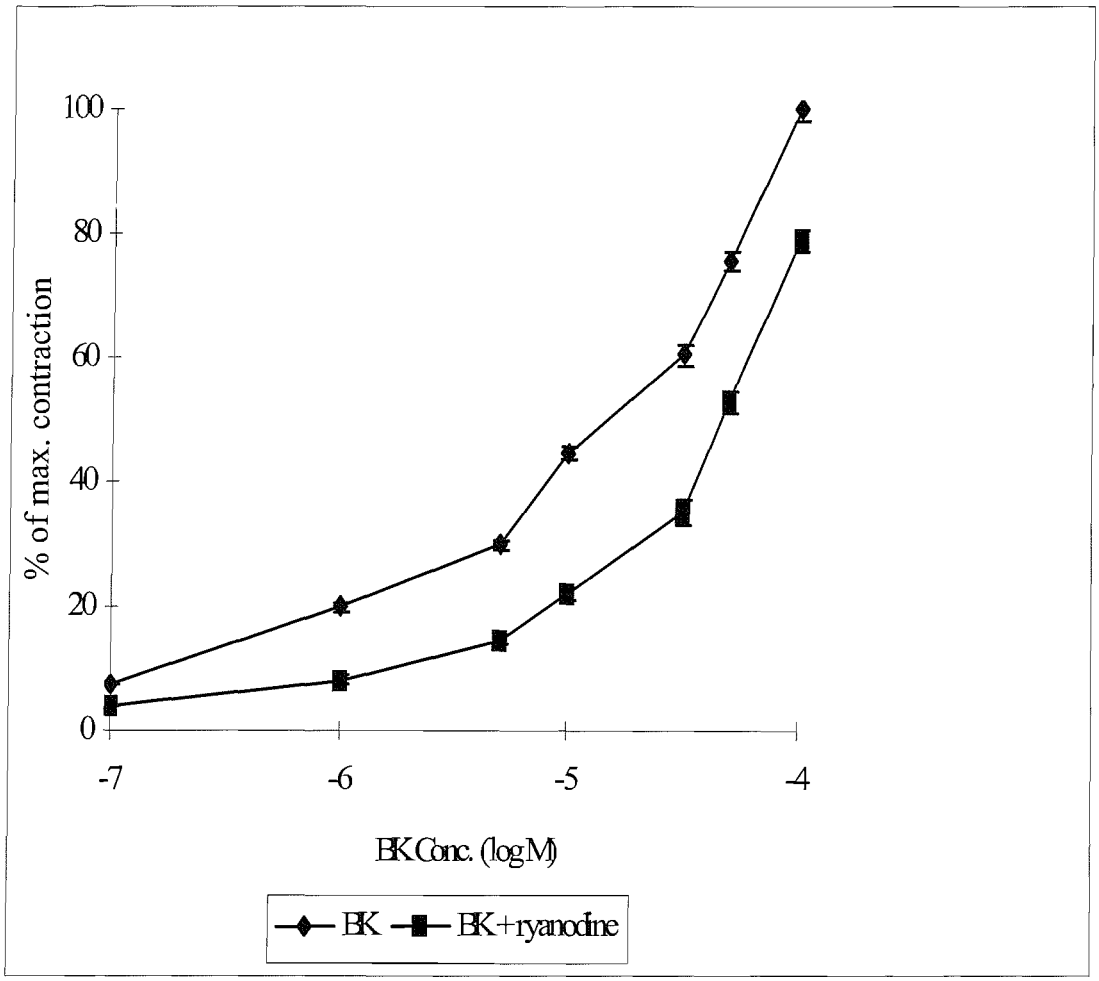


Fig 4.25.The importance of intracellular calcium on BK induced contraction of human gallbladder tissue *in vitro* was examined. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E. mean of 7 experiments. BK concentration response curves (CRCs) were constructed in the absence or in the presence of ryanodine (10μM) $_{P}$KB= 5.93. The data expressed above and the $_{P}$KB values were calculated as previously stated.

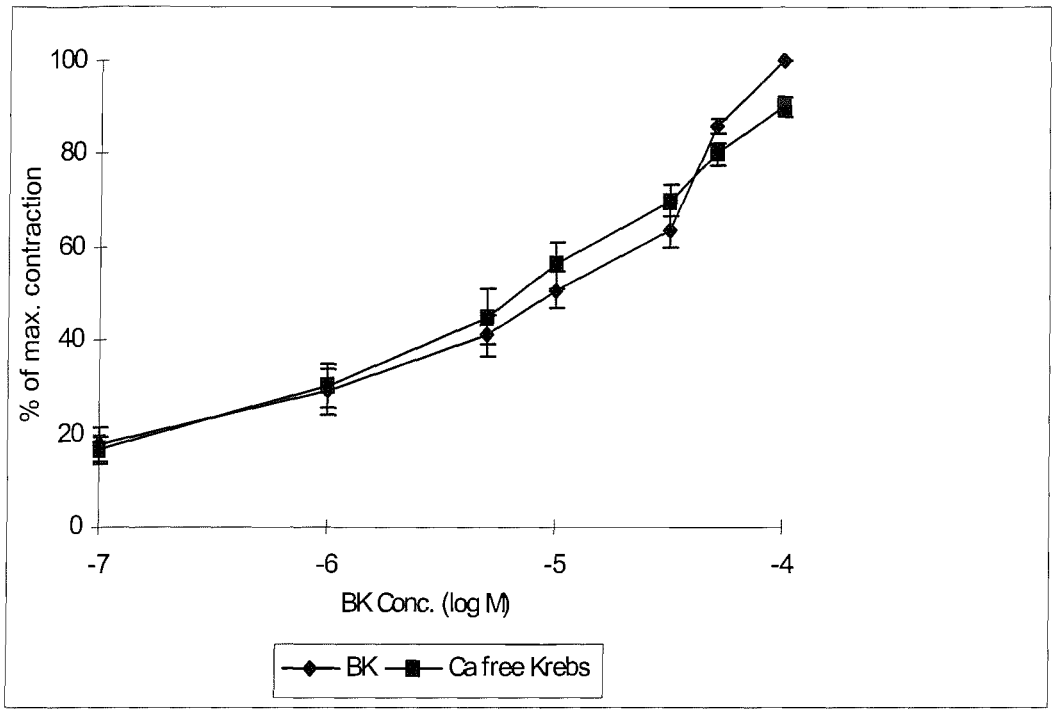


Fig 4.26 The effect of extracellular Ca^{+} on BK induced contraction of human gallbladder tissue *in vitro* was examined. Responses were obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM) in krebs solution ± calcium. The calcium free krebs solution contained EGTA. Each point represents the mean ± S.E. mean of 7 experiments. BK concentration response curves (CRCs) were constructed in the absence or in the presence of extracellular calcium. The data expressed above was calculated as previously stated.

The **arachidonate metabolites** and the arachidonic pathway were previously discussed. Non-steroridal anti-inflammatory drugs (NSAIDs) have three levels of action, all of which are due to, mainly, the inhibition of the arachidonic acid cyclo-oxygenase and the resultant decrease in prostanoid synthesis. The three actions, which have been previously discussed, are the anti-inflammatory action, an analgesic effect and an anti-pyretic effect. Indomethacin is one of the most potent inhibitors of the cyclo-oxygenase pathway *in vitro*. A BK response curve (0.1-100μM) was established and was then repeated in the presence of indomethacin (3μM) – figure 4.27 a/b. A significant rightward shift of the curve was achieved in both normal (figure 4.27a, ANOVA P=0.001, F= 25.069) and stone-bearing (figure 4.27b, ANOVA P=0.002, F= 12.628) gallbladder tissue – as result the BK response is mediated by the cyclo-oxygenase pathway in both normal and stone-bearing gallbladder strips.

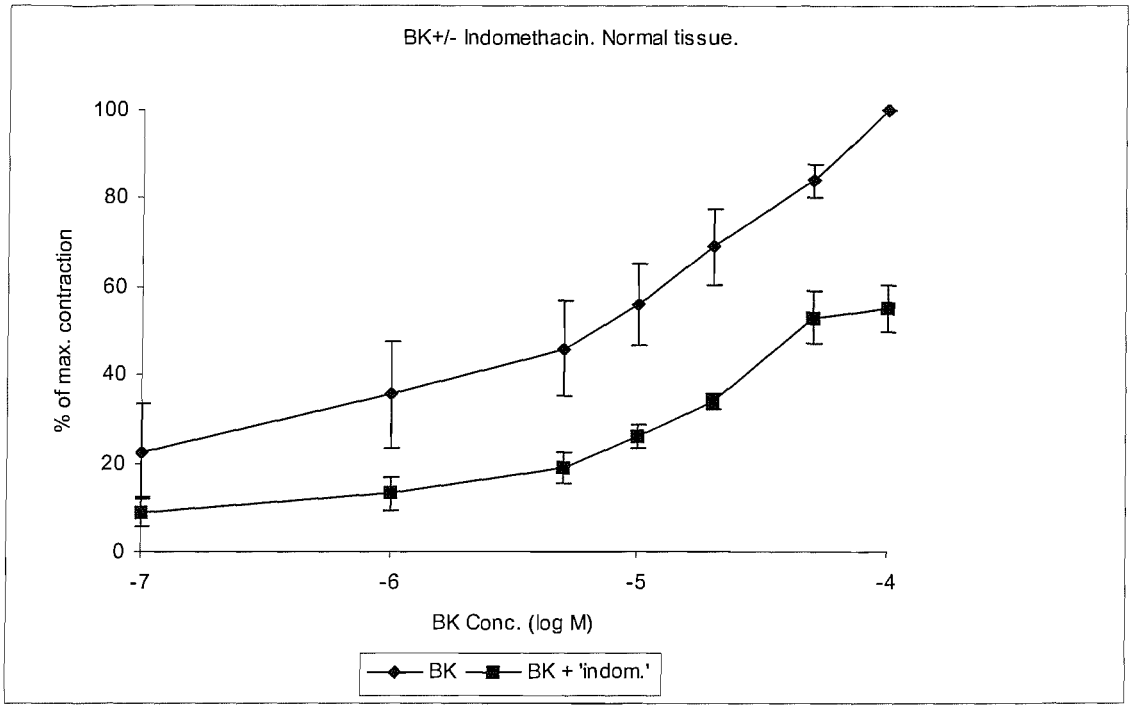


Fig 4.27a.The effect of a cyclo-oxygenase inhibitor on BK induced contraction of normal human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E. mean of 4 experiments. BK concentration response curves (CRCs) were constructed in the absence or in the presence of indomethacin (3μM). $_{P}$KB= 6.02. The data expressed above and the $_{P}$KB values were calculated as previously stated.

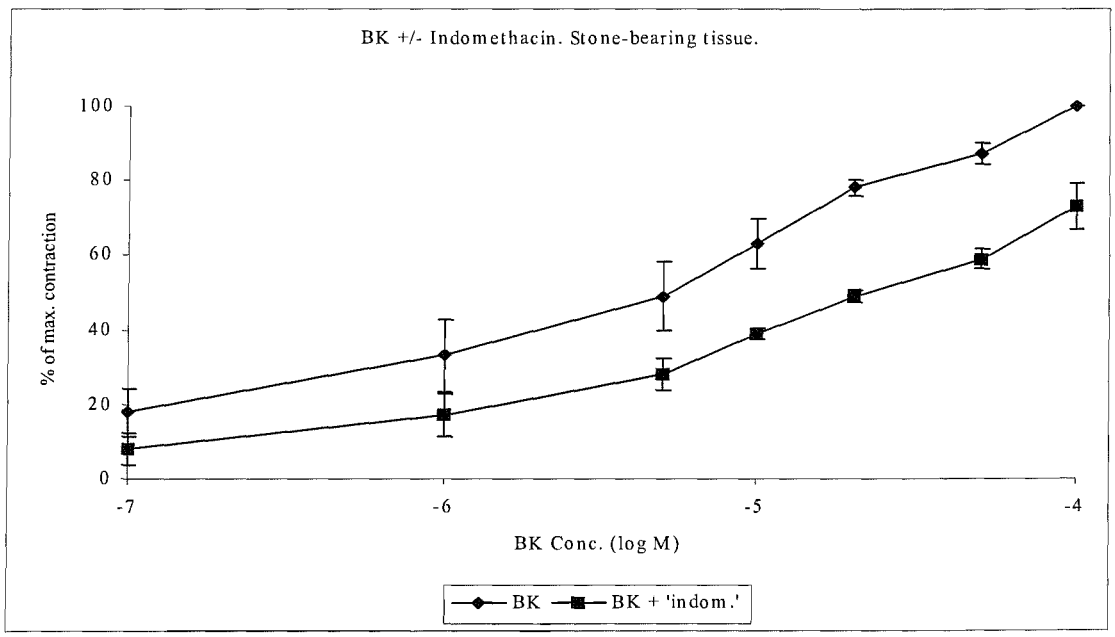


Fig 4.27b.The effect of a cyclo-oxygenase inhibitor on BK induced contraction of stone-bearing human gallbladder tissue *in vitro*. Responses obtained after two hours incubation. Each strip was exposed to cumulative increases of BK (0.1-100μM). Each point represents the mean ± S.E. mean of 4 experiments. BK concentration response curves (CRCs) were constructed in the absence or in the presence of indomethacin (3μM). $_{P}$KB= 6.02. The data expressed above and the $_{P}$KB values were calculated as previously.

4.3 In vivo V In vitro argument

CCK was measured in forty-three individuals : fifteen normal controls and twenty-eight gallstone patients. The gallstone patients were split into two groups, good contractors (n=8) with >50% reduction in gallbladder volume after a meal and poor contractors (n=20). Poor contracting gallstone patients had higher mean peak CCK levels (4.51 $\pm$ 0.48pM, mean $\pm$ S.E.M) than healthy individuals (3.59 $\pm$ 0.29pM). No significant difference was noted in serum CCK levels between normal individuals and good contractors with gallstones.

Statistical analysis

Data are presented as mean and $\pm$ SEM. Tests for statistical significance were performed using the t-test. $P<0.05$ or less was considered as indicative of significance.

A number of the patients above proceeded to have their gallbladders removed and BK and/or CCK dose response curves were obtained as previously described (section 3.3.6). The results were also obtained as previously described (section 3.3.8). Four poor-contracting patients (A1-A4) and two good-contracting patients (B1-B2) were used in this part of the study.

It can be noted from the tables below that it takes a higher concentration of either BK or CCK to contract poor contracting tissue when compared to good contracting tissue and this is indeed what corresponded to what the assay results were i.e. poor contracting gallstone

patients had higher peak CCK levels than healthy individuals and that no significant difference was noted in serum CCK levels between normal individuals and good contractors with gallstones. The results and implications are discussed in the next section.

BK Conc. Patient	0.1uM	1uM	5uM	10uM	20uM	50uM	100uM
A1	1.8	11.7	24	47	68	95	100
A2	1.6	3.2	8.0	19	53	69	100
A3	2.3	4.6	21.4	25.0	42	71.4	100
A4	5.0	11.5	14.8	19.2	27.1	59.2	100
Mean	2.675	7.75	17.05	27.55	47.5	73.65	100

Table 4.3.1 Poor-contracting gallstone patients with BK dose response curve.

CK Conc Patient	10nM	20nM	50nM	100nM	200nM	500nM	1000nM
A1	0.8	1.0	1.6	17	28	60	100
A2	0.9	2.4	6	17	30	60	100
A3	1.2	3.0	5	21	41	72	100
A4	3	5	8.1	33	52	81	100
Mean	1.475	2.85	5.175	22	37.75	68.25	100

Table 4.3.2 Poor-contracting gallstone patients with CCK dose response curve.

BK Conc. Patient	0.1uM	1uM	5uM	10uM	20uM	50uM	100uM
B1	10	26	50	69	79	93	100
B2	16	28	42	60	80	90	100
Mean	13	27	46	64.5	79.5	91.5	100

Table 4.3.3 Good-contracting gallstone patients with BK dose response curve.

CK Conc. Patient	10nM	20nM	50nM	100nM	200nM	500nM	1000nM
B2	10.5	12.8	18	69	89	96	100
	10.5	12.8	18	69	89	96	100

Table 4.3.4 Good-contracting gallstone patients with CCK dose response curve. No CCK results available for B1.

BK Conc.	0.1uM	1uM	5uM	10uM	20uM	50uM	100uM
Mean (PC)	2.675	7.75	17.05	27.55	47.5	73.65	100
Mean (GC)	13	27	46	64.5	79.5	91.5	100

Table 4.3.5 Mean of poor (PC) and good (GC) -contracting gallstone patients with BK dose response curves.

CK Conc.	10nM	20nM	50nM	100nM	200nM	500nM	1000nM
Mean (PC)	1.475	2.85	5.175	22	37.75	68.25	100
Mean (GC)	10.5	12.8	18	69	89	96	100

Table 4.3.6 Mean of poor (PC) and good (GC) -contracting gallstone patients with CCK dose response curves.

5. DISCUSSION

This thesis attempts to investigate the effect of bradykinin (BK) on human gallbladder muscle *in vitro* and to investigate possible mechanisms that mediate the responses. These mechanisms are important as gallbladder motility may influence gallstone formation. Impaired gallbladder emptying prolongs residence of bile in the gallbladder, thus allowing more time for nucleation of cholesterol crystals from supersaturated bile. Furthermore in the case of adequate gallbladder emptying, cholesterol crystals that have nucleated are ejected to the duodenum whereas, in the case of impaired gallbladder emptying, these crystals aggregate into macroscopic gallstones (Van Erpecum and Van Berge-Henegouwen 1999). Although, BK is usually thought of as an inflammatory mediator it has substantial activity on intestinal smooth muscle. Inflammation of the gallbladder is a usual consequence of the presence of gallstones, raising the possibility of excessive BK release in stone bearing gallbladders. With this in mind it is important to investigate any possible connection between the inflammatory mediator BK and any changes in gallbladder motility.

I have examined the effects of BK on human gallbladder tissue *in vitro* using selective BK receptor agonists and antagonists. It has been shown that both types of tissue (normal and stone-bearing) contract in a dose dependent manner in response to BK (fig 4.1) and the study suggests the co-existence of both BK1 and BK2 receptors in both normal and stone-bearing gallbladder muscle tissue (figs 4.3-4.6,4.9). When the BK1 receptor was blocked, by the introduction of des-Arg^{9} -Leu^{8}- BK, a significant rightward shift of the curve was observed in normal gallbladder strips (fig 4.3) but not in stone-bearing gallbladder strips (fig 4.4). BK2

receptor blockade produced a significant rightward shift of the curve in normal and stone-bearing gallbladder strips (figs 4.5-4.6). In stone-bearing gallbladder the response is mediated predominantly via BK2 receptors and the BK1 response seems to be lost. This seems to suggest that a difference in BK receptor population exists between normal and stone-bearing gallbladder tissue. This may be due to an up-regulation of BK2 receptors, or a down regulation of the BK1 receptors, in stone-bearing tissue is unclear. When both the BK1 and BK2 receptors were blocked simultaneously there was no significant additive effect in normal or stone-bearing tissue. That is, the maximal response and ED_{50} remained unchanged suggesting the co-existence of a third bradykinin receptor or a bradykinin independent mechanism – which is discussed later.

Further evidence suggesting a difference in receptor population between the two tissue types is that the selective BK1 receptor agonist, des-Arg^9-BK showed a higher affinity in normal tissue (fig 4.9). A concentration response curve was obtained for a BK1 receptor agonist (des-Arg^9-BK) in both normal and stone-bearing gallbladders. When the BK1 receptor antagonist was present, the only significant rightward shift of the curve was in normal tissue (fig 4.9).

These findings (i.e. different BK receptor population in the same type of tissue) are different to findings in other tissues and other species. However, there is evidence that there is a different function for each receptor type. Lecci et al (1999) stated that stimulation of bladder BK1 receptor evokes a local, tonic-type concentration with reflex contractions superimposed in both normal and inflamed bladders, but in the latter situation the motor responses are magnified. A difference between normal and inflamed tissue does exist: Hall 1997, concluded

that a role for BK2 receptor does exist in more classical acute inflammatory events, such as edema and inflammatory pain whereas the BK1 receptor appears to be involved in chronic inflammatory responses, such as certain forms of persistent hyperalgesia. Moreover, Phagoo et al 1999 noted that at sites of inflammation elevated formation of BK was evident. They also concluded that BK2 receptors mediate the action of BK in the acute stages of inflammation and during the pain response and the BK1 receptors partake in the chronic stage. They hypothesized that kinins autoregulate the BK1 and BK2 receptor expression in favour of the BK1 receptors. These reports along with others (Bhoola et al 1997, Naciaker et al 1999) show that the BK1 receptors expression is more 'prominent' in inflamed tissue while the BK2 expression is either reduced or remains the same as in normal tissue.

The findings reported here suggest that the BK1 receptor population seems to be more 'prominent' in normal tissue than stone-bearing gallbladder tissue. This could result from an autoregulation of the BK receptors in gallbladders that favour the expression of BK2 receptors in stone-bearing tissue. The actual presence of the gallstone may result in a down regulation of the BK1 function as other mediators are called into action. Meini et al. (1998) suggest that there is a difference between the BK receptor populations in normal and inflamed gallbladder muscle and because the contraction response of the inflamed tissue is not reduced by COX inhibitors. They argue that an additional mechanism plays a compensatory role in inflamed tissue. A similar mechanism could be at work in stone-bearing inflamed gallbladders, which has the result of either reducing the BK1 receptor population or the BK1 activity. A recent study (Banik et al 2001) concluded that in chronically inflamed tissue, nociception to BK, which is BK2 receptor mediated, is strongly increased and that the BK1 receptor may be not

important to a persistent inflammatory state. The presence of gallstones in the gallbladders in this study does represent a persistent inflammatory state – which may result in 'a less important role' for the BK1 receptor when compared to the 'normal' state. Another example of a more prominent role for the BK2 receptor in the pathological state can be found with the portal hypertensive response to BK in inflamed or cirrhotic rat livers. BK2 receptors, but not the BK1 receptors mediate this response, and there is a contracting hyporeactivity to BK in cirrhotic rat livers. The possibility of the involvement of other mechanisms was examined in this project and will be discussed later in this section.

The difference between the present results and other reports (concerning the BK1 population) may also involve the argument regarding the formation of gallstones: does the presence of gallstones in the gallbladder precede inflammation or does inflammation result in gallstone formation? As the results conflict with other reports with inflamed tissue, mentioned above, it might be that gallstone formation appears first followed by inflammation and therefore the BK receptor population in gallbladder tissue is unlike other tissue. These examples show that a difference may exist for the function of BK1 and BK2 receptors.

There is also considerable evidence that a species difference does exist concerning the cloning expression and pharmacological comparison of human and other species BK receptor types. Hess et al. (1992) concluded that the human and mouse BK2 receptor genes indicate that some of the previous reports of BK2 receptor subtypes can be explained by species differences in a single BK2 receptor gene. Regoli et al 1994 compared BK receptor types in rat, guinea pig, rabbit and man, and confirmed the existence of two BK2 receptor subtypes. They suggest that

receptor subtypes may be species dependent and indicated that the BK2 subtype found in rabbit is similar to that found in man. So there are examples of BK receptor expression that are similar between species but also examples of inter and intra species differences. Paquet et al.1999 suggested that inter-species differences should be assumed when setting up animal models with the aim of developing BK2 receptor antagonists as therapeutic agents.

The results in this study show that high concentrations of BK cause contraction, even in the presence of BK1 and BK2 receptor antagonists, suggesting the co-existence of another BK receptor or a receptor independent mechanism. Farmer et al (1989) suggested that pulmonary tissue, particularly in the large airways contains a novel BK3 receptor which may be involved in BK induced bronchoconstriction. Farmer et al (1991) went on to observe that BK-induced efflux of calcium ions was unaffected by BK1 or BK2 antagonists, this provided further evidence that airway smooth muscle contain a novel BK3 receptor. Field et al 1992 proposed that the novel BK3 receptor might be in part species dependent. Farmer and DeSiato (1994) provided the additional evidence for heterogeneity of BK receptors within the same species (guinea-pig) and, furthermore, indicate that the tracheal BK (BK3) receptor is different from that in ileal tissue. Several studies have given conflicting results and no clear-cut conclusions can be made regarding the existence or non-existence of BK3 receptors (Hall and Morton 1997). The alternative explanation to the finding is that the high BK concentration overcomes the competitive inhibitors but this seems unlikely given the large differences in receptor affinity of the two antagonists and the similar slope of the concentration response curve in the presence of each antagonist in normal tissue. In addition increasing concentrations of the two antagonists had no further inhibitory effect on a sub-maximal dose of BK.

Cholecystokinin (CCK) is one of three classical gut hormones and was named for its ability to cause gallbladder contraction (Ivy and Oldberg 1928). The factors that control the release of CCK are complex and not fully understood. CCK may be involved in the pathophysiology of several gastrointestinal disease processes. As CCK is such an important hormone in gallbladder motility, an investigation into any possible relationship between the mechanisms of action of CCK and BK was explored. Both normal and stone-bearing gallbladder strips contracted in a dose-dependent fashion to CCK as expected. On addition of the BK1 receptor antagonist a rightward shift of the curve was noted with normal but not with stone-bearing gallbladder strips. A rightward shift of the curve was achieved with both types of tissue, when the CCK curve was repeated in the presence of the BK2 receptor antagonist. This suggested that the CCK response may be mediated through both BK receptors in normal tissues but only through the BK2 receptor in stone-bearing tissue. The BK receptors seem to partly mediate the CCK response; a BK concentration response curve was repeated in the presence of a known CCK receptor antagonist. No significant effect on the curve was noted in either normal or stone-bearing tissue. It can be stated therefore, that the CCK contraction response was in some way mediated by the BK receptor mechanism but because there was no rightward shift of the BK curve in the presence the CCK receptors antagonist it also can be stated that the CCK receptors did not mediate the BK response.

It was established that BK-induced contraction was inhibited by indomethacin - which is a potent inhibitor of cyclo-oxygenase inhibitor *in vitro*. The BK response was not affected by pyramine (a histamine receptor antagonist), atropine (a muscarinic receptor antagonist), prazosin (an adrenoceptor blocker), tetrodotoxin (a Na channel blocker), quinidine (a K

channel blocker) and the BK response was also not affected in Ca free medium. The data suggest that the BK response depends on the release of intracellular Ca and that the contraction is mediated by the release of prostanoid metabolites derived from the cyclo-oxgenase pathway from arachidonic acid as the BK response was inhibited by indomethacin. This was similar to a finding by Ikeda et al (2001) – where inflammatory pain was induced following an intradermal injection of carrageenin into rat paws. The results indicated that BK, acting on the BK2 receptor, is the main mediator at the early phase of inflammatory pain of carragenin oedema and that pre-treatment with indomethacin (cyclo-oxygenase inhibitor) inhibited the carrageenin-induced responses significantly. The actions of BK in this study are also realised through the cyclo-oxygenase pathway.

The question as to what extent the results obtained in any *in vitro* study can be compared to what happens *in vivo* is always posed. An attempt was made in this study to address that question. A number of patients in this study had three sets of data – ultrasound findings, CCK assay levels and dose response curves *in vitro*. Ultrasound findings split the gallstone patients into two groups, good contractors (n=8) with >50% reduction in gallbladder volume after a meal and poor contractors (n=20). Poor contracting gallstone patients had higher peak CCK levels (4.51 $\pm$ 0.48pM mean $\pm$ S.E.M) than healthy individuals (3.59 $\pm$ 0.29pM). No significant difference was noted in serum CCK levels between normal individuals and good contractors with gallstones. These facts mirror what happens *in vitro* and the results can be compared (see tables 4.3.5/6) i.e. it takes a higher concentration of either BK or CCK to contract poor contracting tissue when compared to good contracting tissue. e.g. at 10nM CCK the contraction in poor contracting tissue is only 1.48% when compared to good contraction

tissue (10.5%) and at 500nM the contraction in poor contracting tissue is only 68.25% when compared to 96% in good contracting tissue. These results are interesting and give and insight to what conclusions can be drawn between *in vitro* and *in vivo* work. The question is always there when doing *in vitro* work – how will this compare physiologically ? This work answers it to a degree and states that the results are comparable. Yet still questions shall remain as doses used *in vitro* are not comparable to doses *in vivo.* When extrapolating *in vitro* situations, one should be extremely careful with such things as the therapeutic concentrations of the studied substance in relation to the in vitro substrate concentration and inhibitory values. For example 100nM of CCK was added to each gallbladder strip in some of the experiments in this project on a continued basis, it remains unknown what affect this would have *in vivo* as the strips in this project were isolated. Nevertheless the results indicated here did show that there was a parallel between the two sets of results. The elevated levels of CCK seen in poor contracting patients confirm the results of previous studies and may be due to a lack of positive feedback inhibition on the neurohormonal axis of control of gallbladder emptying arising from decreased volume of bile entering the duodenum.

Possible future work

The investigation and definite proof of a further class of BK receptor requires molecular biological techniques. The advances made in the BK receptor pharmacological front as described in this thesis and in other findings have resulted from the use of effective BK receptor antagonists. The research described in this thesis suggests that there is an *in vitro* response to BK in human gallbladder that cannot be suppressed by either the BK1 receptor antagonist or the BK2 receptor antagonist or a combination of the two. This is true even for a

vast excess of the 2 compounds, both of which are considered to be complete blockers of BK activity. Despite the advances made in this project a definite receptor population using only pharmacological identification is considered too inaccurate as the BK may become masked by the presence of the BK1 and BK2 receptors. Similarly cloning by crosslinking any of the known antibodies to the receptor may result in many false positives from BK1 and BK2. This would prove extremely expensive as each protein would have to be analysed and sequenced. As stated above molecular biology is the way forward to determine what BK receptor populations are actually involved and to confirm the possible existence of other BK receptors. All gallbladder tissue used in this project has been snap frozen in liquid nitrogen so the possibility does exist for molecular work - extract mRNA from all of these specimens for RT-PCR to determine expression of both BK receptors. Each segment would then be analysed for expression of BK receptor like sequences using low stringency Northern blotting and degenerate RT-PCR. Primers and probes designed for these techniques would be based on regions of homology between known BK receptor RNAs. Any PCR products would be cloned and sequenced, novel sequences could be used as probes on a gallbladder cDNA library to clone a full length cDNA. Differences in BK1 and BK2 responses in healthy and stone-bearing gallbladders should aid identification of the relevant cDNA. Once identified the new sequence used to identify novel compounds for the treatment of gastrointestinal disorders, especially gallstones and gallstone induced pancreatitis.

An understanding of the relationship between inflammatory mediators and the control of gallbladder contraction will improve our understanding of the mechanisms of gallbladder formation and the pathogenesis of the symptoms. The future will incorporate the

pharmacological evidence, as in this thesis, combined with the molecular evidence, described above, and that shall increase our knowledge of BK receptors and from these studies specific BK receptors and their genes may target the development of therapeutically viable drugs.

Summary

In conclusion, agonist and antagonists have been used as pharmacological tools to investigate the role of BK on normal and stone-bearing tissue. The experiments have shown that:

1. both types of tissue contract in a dose-dependent manner to BK.
2. both the BK1 and BK2 receptors are involved in this action.
3. a difference in the BK receptor population seems to exist between the two types of tissue.
4. a third BK receptor or a BK independent system may exist.
5. BK receptors did mediate the CCK response.
6. The BK response was not mediated by the CCK receptor mechanism.
7. The BK response requires the release of intracellular calcium.
8. The BK response is mediated by the release of metabolites derived from the cyclo-oxygenase pathway from arachidonic acid.
9. There is a correlation between the *in vivo* and i*n vitro* work.

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