CHAPTER 2

LITERATURE REVIEWS

Acute and chronic diarrhea, caused by many microorganisms including some parasites, has been of major concern to human health since the earliest recorded history. Bacteria such as *Escherichia coli*, *Vibrio cholera*, *Shigella* and *Salmonella* are always reported to cause diarrhea throughout the world (Rhodes and Tsai, 1995). Intestinal protozoa, especially *Entamoeba histolytica*, is also one of a common cause of acute and chronic diarrhea, particularly in developing countries (Walsh, 1986) and areas with immigrants from Mexico, Central America and Southeast Asia (Maltz and Knauer, 1991). The clinical spectrum of illness may range from asymptomatic carriage to invasive disease with formation of liver abscesses.

*E. histolytica* was first documented by Losch in 1875 as pathogenic protozoa (Ravdin, 1995). It is a pseudopod-forming protozoan within the subphylum Sarcodina, superclass Rhizopoda, class Lobosea, order Amoebidae and family Entamoebidae (Levine, Corliss and Cox, 1980). It has two forms: trophozoite (Figure 1) and cyst. Trophozoites are usually actively motile. They move by means of pseudopodia, cytoplasmic protrusion that may be formed at any point on the surface of the organism. The pseudopodium is quickly thrust out and may vary in form from short, blunt, and broad, to long and fingerlike. Living trophozoites vary in size from about 12 to 60 µm in diameter. Cyst is usually spherical but may be ovoid or irregular in shape and they vary from about 10 to 20 µm in diameter. Infection is initiated by ingestion of food or water contaminated with fecal matter containing mature cysts. Excystation into the trophozoite form occurs in the lower small bowel, and the trophozoites are capable of penetrating into colonic mucosa. Trophozoites encyst in the colon, and
these cysts are excreted in the stool, releasing infectious cysts into the environment to renew the life cycle. In some cases, trophozoites invade through the colonic mucosa and reach the portal circulation, where they penetrate into liver tissue and create an amoebic liver abscess (Stanley, 1997).

![Entamoeba histolytica trophozoite](image)

**Figure 1** *Entamoeba histolytica* trophozoite

**Epidemiology**

Epidemiology surveys for infection with *E. histolytica* are difficult to interpret owing to the low number of infected patients who demonstrated the organism on a single stool examination (Mathur and Kaur, 1973; Healy, 1988), frequent laboratory error identification (Krogstad, Spencer and Healy, 1978), and the variability of detection of serum anti-amoebic antibodies after infection (Ravdin and Petri, 1995). It is estimated that more than 10% of the world's population is infected (Walsh, 1988). In Oakland, California, Markell and Kuritsudo (1981) found a prevalence rate of 0.7% in asymptomatic persons surveyed as part of a routine health examination, however, in some areas such as Gambia, West Africa (Bray and Harris, 1977), Bangladesh (Hossain *et al.*, 1983) and India (Choudhuri *et al.*, 1991) infection rate is as high as 50%. Persistence of a high prevalence of
amoebic infection depends on cultural habits, sanitation, crowding, and socioeconomic status (Bray and Harris, 1977; Abdel-Hafez, el-Kady and Bolbol, 1985). Asymptomatic intestinal infection occurs in 90-99% of infected individuals (Nanda, Baveja and Anand, 1984; Jackson, Gathiram and Simjee, 1985; Jackson, 1987), in most cases the parasite was eliminated from the gut within 12 months without treatment (Nanda, Baveja and Anand, 1984). In highly endemic area in Durban, South Africa, a 10% prevalence of *E. dispar*, the protozoa morphology identical to *E. histolytica*, was found in asymptomatic cases (Ravdin and Petri, 1995) and *E. histolytica* infection resulted in 0.1% prevalence of amoebiasis each year (Gathiram and Jackson, 1985; Gathiram and Jackson, 1987).

**Pathology**

*Entamoeba histolytica* naturally infects only humans and perhaps some higher non human primates (Stanley, 1997). The disease is usually gradual, with abdominal pain and discomfort associated with frequent bowel movements. Rectal pain and urgency to defecate are also common. The stool is often noted to be loose, watery and contain varying amounts of blood and mucus. Patients may have anywhere from a few bowel movements per day to several movements per hour. The combination of bloody diarrhea, abdominal pain, and urgency to defecate are the classic presentation of amoebic dysentery (Shulman *et al.*, 1997). A spectrum of colonic lesions ranging from nonspecific thickening of the mucosa to the classic flask-shaped ulcer may be associated with amoebic infection (Prathap and Gilman, 1970; Pittman, El-Hashimi and Pittman, 1973). Complications include perforation of the intestine, leading to peritonitis, and extra-intestinal invasion. Trophozoites can spread via the blood to the liver, with the formation of an abscess, and may secondarily extend to the lung and other organs. Rarely, abscess spread directly and involves the overlying skin. *E. histolytica* exerts a lytic effect on tissue, a characteristic for which the organism is named. Reports of initial invasion of amoebae via mucosal
crypts have not been confirmed. Amoebae appear to invade the colonic epithelium directly (Brandt and Perez Tamayo, 1970; Griffin and Juniper, 1971). Light and electron microscopic studies showed mucosal cells lysis on contact with amoebae or, alternatively, diffuse mucosal damage before amoebic invasion (Griffin and Juniper, 1971; Pittman, El-Hashimi and Pittman, 1973; Takeuchi and Phillips, 1975). An amorphous, granular, eosinophilic material surrounds trophozoites in tissue, whether in colon, liver, lung or brain (Chatgidakis, 1953; Brandt and Perez Tamayo, 1970; Prathap and Gilman, 1970). Consistent with the fact that trophozoites have the capacity to destroy leukocytes (Guerrant, Brush and Ravdin, 1981; Ravdin, Murphy and Salata, 1985), inflammatory cells are found only at the periphery of established amoebic lesion (Brandt and Perez Tamayo, 1970; Prathap and Gilman, 1970). In vivo and in vitro studies demonstrated that lysis of host neutrophils by E. histolytica results in the release of toxic nonoxidative neutrophil products that contribute to the destruction of host tissue (Tsutsumi, Mena-Lopez and Anaya-Velazquez, 1984; Salata and Ravdin, 1985).

Many species of rodents have been used as models of human amoebiasis such as the guinea-pig (Rees, Taylor and Reardon, 1954), the golden hamster (Neal and Vincent, 1955; Williams, 1959 and Jarumilinta and Maegraith, 1961), weaning rat (Jones, 1946; Taylor et al., 1950, Sudan et al., 1950 and Neal and Harris, 1977) and albino mice (Ray and Chatterjee, 1981). These animals model also useful for drug testing on human amoebiasis (Owen, 1985).

**Drugs used in the treatment of amoebiasis**

Drugs used to treat amoebiasis can be categorized as luminal, systemic or mixed amoebicides (Tracy and Webster, 1996). Luminal amoebicides, exemplified by diloxanide furoate, are active only against intestinal forms of amoebae. These compounds can be used successfully by themselves to treat asymptomatic or mild intestinal forms of amoebiasis, or
in conjunction with a systemic or mixed amoebicide to eradicate the infection. Systemic amoebicides are effective only against invasive forms of amoebiasis. These agents have been employed primarily to treat severe amoebic dysentery (dihydroemetine) or hepatic abscess (dehydroemetine or chloroquine), but they are now rarely used unless other drugs fail or cause unacceptable side effects. Mixed amoebicides are active against both intestinal and systemic forms of amoebiasis. Metronidazole, a nitroimidazole derivative, is a prototypical mixed amoebicide, and its use has revolutionized the treatment of this infection (Tracy and Webster, 1996; Freeman, Klutman and Lamp, 1997; Katzung, 1997). Because metronidazole is well absorbed and therefore may fail to reach the large intestinal in therapeutic concentrations, this compound is likely to be more effective against systemic than intestinal amoebiasis. Antibiotics such as an amoebicidal aminoglycoside, paromomycin, or a tetracycline can be used in conjunction with metronidazole to treat severe forms of intestinal amoebiasis. Treatment with metronidazole is usually followed by luminal amoebicide to affect a complete cure (Tracy and Webster, 1996).

**Metronidazole**

Metronidazole [1-(2-hydroxyethyl)-2-methyl-5nitroimidazole] was discovered in the late 1950s when researchers at Rhone-Poulenc Research Laboratories in France were trying to create azomycin, a synthetic product from a *Streptomyces* spp. This compound is now accepted to be the drug of choice for the treatment of many form of amoebiasis as mentioned above (Cosar and Julou, 1959).

The chemical structure of this compound is shown in Figure 2.
**Figure 2** Structural formula of metronidazole.

**Mechanism of action**

Metronidazole is primarily active against obligate anaerobic microorganisms, both bacteria and protozoa. The 5-nitro group undergoes reductive transformation to an active intermediate which then exerts an inhibitory or lethal effect against DNA (Muller, 1981; Sigeti, Guiney and Davis, 1983). Not only is DNA synthesis inhibited but the reduced metabolite also causes a loss of the helical structure of DNA with subsequent DNA strand breakage. The structure of this intermediate has not been determined. Other reduction-oxidation processes within anaerobic organisms may also inhibit the DNA synthesis (for example, the phophoroclastic reaction in clostridia) (Lockerby, Rabin and Laishley, 1985), which also contribute to cell death.

**Antiparasitic and antimicrobial effects**

Metronidazole is active against a wide variety of anaerobic protozoal parasite and anaerobic bacteria. The compound is directly trichomonicidal. Sensitive isolates of *Trichomonas vaginalis* are killed by less than 0.05 μg/ml of the drug under anaerobic conditions (Johnson, 1993). The drug also has potent amoebicidal activity against *E. histolytica* grown in culture by itself or in mixed culture conditions (Burchard and Mirelman, 1988). Trophozoites of *Giardia lamblia* are directly affected by metronidazole at concentrations of 1 to 50 μg/ml *in vitro* (Jokipiï and Jokipiï, 1980). Metronidazole manifests
antibacterial activity against all anaerobic cocci and both anaerobic gram-negative bacilli, including *Bacteroides* species, and anaerobic spore-forming gram-positive bacilli. Non spore-forming gram-positive bacilli are often resistant, as are aerobic and facultative anaerobic bacteria.

**Therapeutic use for amoebiasis**

Metronidazole is an effective amoebicide and has become the agent of choice for the treatment of all symptomatic forms of amoebiasis (Tracy and Webster, 1996; Freeman, Klutman and Lamp, 1997; Katzung, 1997). In all geographic areas and regardless of the virulence of the parasite or the form of infection being treated, it is recommended that patients receive 750 mg of metronidazole, three times daily for 5 to 10 days. The daily dose for children is 35 to 50 mg/kg, given in three divided doses for 10 days. Treatment with metronidazole is the least effective when the drug is administered to an asymptomatic passer of cysts, probably because absorption occurs higher in the gastrointestinal tract. Although metronidazole is still effective, fewer failures results from the use of purely luminal amoebicides, such as diloxanide furoate in asymptomatic infection. The latter are thus preferred alone or in combination with metronidazole in such an infection (Tracy and Webster, 1996).

**Adverse effects of metronidazole**

The toxicity of metronidazole has been reviewed by several authors (Roe, 1977; Lau *et al.*, 1992). Side effects of metronidazole occur rarely and mostly are not severe enough to discontinue the therapy. The most common side effects are headache, nausea, dry mouth, and a metallic taste. Vomiting, diarrhea, and abdominal distress are occasionally experienced. Furry tongue, glossitis, and stomatitis may occur during therapy and are associated with a sudden intensification of moniliasis. Dizziness, vertigo, very rarely encephalopathy, convulsions, incoordination, and ataxia are neurotoxic effects that warrant discontinuation of metronidazole. The drug
also should be withdrawn if numbness or paresthesia of the extremities occurs. Reversal of serious sensory neuropathies may be slow or incomplete. Urticaria, flashing, pruritis, dysuria, cystitis and a sense of pelvic pressure also have been reported. Metronidazole has a well-documented disulfiram-like effect, such that some patients experience abdominal distress, vomiting, flushing or headache if they drink alcoholic beverages during therapy with this drug. Patients should be cautioned to avoid consuming alcohol during metronidazole treatment, even though the risk of severe reaction is low. In the same vein, concurrent administration of metronidazole and disulfiram is not recommended, because confusion and psychotic states may occur. Although related chemicals have caused blood dyscrasias, only a temporary neutropenia, reversible after discontinuation of therapy, occurs with metronidazole (Lau et al., 1992).

Metronidazole should be used with caution in patients with active disease of the CNS because of its neurological toxicity. The dosage should be reduced in patients with severe obstructive hepatic disease, alcoholic cirrhosis or severe renal dysfunction (Lau et al., 1992).

Metronidazole and some of its metabolites have been shown to be mutagenic in certain bacterial test systems (Vood, van der Stel and Jacobs, 1974; Cornner et al., 1977), along with the reported occurrence of metronidazole resistance to human pathogenic bacteria, *Helicobacter pylori* (van Zwet et al., 1994). The basis for the mutagenic effect (and antimicrobial effect) appears to be dependent upon the reduction of the nitro group, which normally would not occur to any significant degree in normal mammalian cells (Bost, 1977). It could possibly occur in very hypoxic or neurotic tissue. The tumorigenicity of metronidazole has been demonstrated in certain laboratory animals (Rustia and Shubik, 1972), but not in humans, although it should be noted that metronidazole does induce DNA single-strand breakage in the lymphocytes of patients on standard dose of the drug (Reitz, Rumpf and Knitza, 1991).
suppressed the humoral immune response. Cell-mediated immunity was also suppressed by metronidazole as observed from the T-cell counts and the leukocyte migration inhibition (LMI) test. The immunosuppressive effect of metronidazole, however, appeared to be transient as the immune functions returned to normal within 15 days. Similarly, these side effects were obtained in previous immune modulation studies (Saxena et al., 1985). Moreover, there is no firm evidence for teratogenicity or embryotoxicity with metronidazole in animals or in humans (Morgan, 1978). At dose levels approximately 5-10 times those used in humans, metronidazole caused microscopic changes in the liver in monkeys and central nervous system effects (ataxia, tremors and prostration) in dogs (Bost, 1977). Evidence that caused concern regarding cytogenic effects has been corroborated (Roe, 1979).

Medicinal plants

Current drugs used for treatment of amoebiasis are stilled problems and have some limited. For example, emetine and dihydroemetine have significant side effects, including arrhythmia, congestive heart failure with dyspnea and hypotension. Serious side effects may limit the use of these medications, particularly in immune-compromised individuals who may be required to take them for long period of time. Furthermore, emetine and dihydroemetine should not be used in patients with cardiac or renal disease, a recent history of polyneuritis, or in young children and in women during pregnancy. For these reasons, it is of interest to search for new compounds which are safer and perhaps more effective than the available drug for the treatment of amoebiasis. Moreover, medicinal herbs and herbal extracts are an indispensable part of the traditional medicine practiced all over the world due to low costs, easy access and ancestral experience.

Many kinds of medicinal plants have been studied for their antiparasitic activities including activities against E. histolytica. The crude
ethanolic extract of *Euphorbia birta* has been shown to eradicate *E. histolytica* *in vitro* (Basit, Siddiquiz and Ahmed, 1977). The ethanolic extract of *Coleus forskohlii* also showed therapeutic efficacy on caecal amoebiasis of rat *in vivo* (Varma et al., 1990). The 50% aqueous ethanolic extract of *Nyctanthes arbor-tristis* was also screened for antiamoebic activity. The extract showed promising effect in clearing *E. histolytica* infections in rat caecum, however, they were not active *in vitro* (Chitravanshi et al., 1992). The ethanolic extracts of mixture of *Boerhavia diffusa, Berberis aristata, Tinospora cordifolia, Terminalia chebula* and *Zingiber officinale* showed therapeutic efficacy against caecal amoebiasis in rats and also demonstrated activity *in vitro* (Sohni, Kaimal and Bhatt, 1995). The similar extracts were also used to test its activity in experimental amoebic liver abscess in golden hamsters. The results showed that the extract was able to cure the amoebic liver abscess (Sohni and Bhatt, 1996). The ethanolic extract of *Piper longum* showed amoebicidal activity against caecal amoebiasis in rats, however, it is not active *in vitro* (Ghoshal, Krishna Prasad and Laksmi, 1996). Water extracts from twenty traditional Chinese medicines have also been studied for their activity on other intestinal protozoa parasite, *Blastocystis hominis, in vitro*. Two of them, *Coptis chinensis* and *Bruea javanica*, were found to be very active against *B. hominis* (Yang et al., 1996). Sengmak (2003) reported *in vitro* effect of some medicinal plants including *P. longum* fruits, *P. sarmentosum* root and *Q. infectoria* nut gall against *E. histolytica*. It is of interest to further study their activities in experimental caecal amoebiasis *in vivo*. The botanical descriptions of the three plants are described in detailed as follows.
Figure 3 Photograph of medicinal plants. (a) *Piper longum* Linn. (b) *Piper sarmentosum* Roxb. and (c) *Quercus infectoria* Oliv.

**Piper longum** Linn.

*Synonyms*: *Piper retrofractum* Vahl.

*Piper chaba* Hunter

*Family*: Piperaceae

*Names (Thai)*: Dee plee, Dee plee chueak.

**Botanical Descriptions**

*P. longum* is a climber; having stem cylindrical, zigzag, glabrous, with a prominent nodes. Leaves are simple, alternative; ovate to oblong; coriaceous; 8.5-16 by 3.5-6.5 cm; consisting of acuminate apex, unequal-sided base; margin entire; base nerves 3-5, the upper nerves are pinnate and curved; petiole 1-1.5 mm long. Flowers are very small in dense spikes, having leaf-opposed, without perianth, each subtended by a very small peltate bract, male and female spike separated; male spike about 5 cm long, containing 2 stamens, the anthers of 2 distinct cells; female spike 3-4 cm long, stigmas 2-3. Fruits are berries, broadly round, 1 seeded. Seed is globes (Farnsworth and Bunyapraphatsara, 1992).

**Ethnomedical Uses:**

The claimed efficacies in Thai traditional text book of *P. longum* Linn. are as follows.
Roots: treatment of paresis, as an antipyretic, antidiarrheal and carminative, antivenin, emmenagogue, abortifacient, headache gout, body pain, rheumatism

Stem: treatment of toothache, stomachache, abdominal discomfort, mucous bloody stools, as an anti-snake venom treatment, expectorant

Leaves: treatment of bodily discomfort, abdominal disease, anemia, phantom tumor, typhoid fever, bronchial asthma

Flower: treatment of nausea and vomiting, abdominal discomfort, bronchial asthma, hemorrhoids, vertigo, viscous saliva, cough, paresis, antidiarrheal

Fruits: treatment of bronchial asthma, bronchitis, muscle pains, as a fire element tonic, emmenagogue and element tonic, antifertility, abdominal pain, vomiting, carminative, heart pain, analgesic, cough, prevent recurrence of asthma, antidiarrheal and dysentery, eye disease, toothache

Not specified part used: for the improvement of blood circulation; treatment of abdominal disease, bronchial asthma, as fire element tonic and element tonic, contraceptive, antifertility

Pharmacological activities

Tripathi et al. (1999) have shown that aqueous and ethanol extract of P. longum fruit strongly active against Giardia lambia both in vitro and in vivo. Both extracts also possessed specific and nonspecific immunostimulatory activity.

Extracts from P. longum fruit also increase bioavailability of drugs, either by promoting rapid absorption from the gastrointestinal tract, or by protecting the drug from being metabolized/oxidized, or by a combination of these two mechanisms (Atal, Zutshi and Rao, 1981).

Piperine, a major alkaloid from P. longum, was able to interrupt pregnancy in 17 of 19 mice when given orally at 50 mg/kg body weight, twice daily on day 2-5 post coitus (Piyachaturawat et al., 1982;
Piyachaturawat *et al.*, 1991), and piperine from *P. longum* and *P. nigrum* has some hepatoprotective potency both *in vitro* and *in vivo* (Koul and Kapil, 1993).

Nadar and Pillai, (1989) reported that the mixture of Ayurvedic medicines (*Glycyrrhiza glabra*, *Terminalia chebula*, *P. longum* and *Shanka bhasma*) improved the secretory status of Brunner's gland involved in the protection against duodenal ulcer.

In an acute and chronic toxicity test in mice, the ethanolic extract of *P. longum* fruit caused non-significant acute or chronic mortality. Furthermore, it induced a significant increase in reproductive organ weight, sperm motility, sperm count and failed to illicit any spermatotoxic effect (Shah *et al.*, 1998).

**Chemical constituents**

The principal substance responsible from the sharp taste of pepper is the alkaloid piperine which has been known through isolation and synthesis for nearly a hundred years. Atal *et al.* (1966) isolated chemical compositions of this plant and found a major alkaloid, pipatine. Chatterjee and Dutta (1966) also examined chemical composition of alkaloid of *P. longum*. According to the report, piperlonguminine was first isolated in 0.002% yield from *P. longum*. Initially its structure was thought to be a piperidine of trimethoxycinnamic acid but the same chemical have been re-isolated, subsequently, given the name piperlongumine (piperlangumine), reanalysed to show a fifth oxygen atom and assigned the structure of piperidone amide (Chatterjee and Dutta, 1966; Chatterjee and Dutta, 1967). A recent report supports a structure with an isomeric double-bond position (Joshi, Kamat and Saxsena, 1968) but encourages a return to the original (and simpler) naming of pipratine. Nigam and Radhakrishnan (1968) studied the chemical constituents of the essential oil of *P. longum*. The essential oil of *P. longum* contained 0.6% yield as a greenish yellow mobile liquid. Chemical analysis
showed that the major constituent of the oil is caryophyllene. Dutta, Banerjee and Roy (1975) identified a light petroleum ether extract of the seed of *P. longum*. The active constituents are sylvatin, sesamin and diaeudesmin. Manavalan and Singh (1979) determined the compounds from the petroleum ether extract of *P. longum* leaves. They are hentriacontane, hentricontane-16-one, triacontanol and β-inositol. The methanol-water (77-23) extract of *P. longum* was examined for the chemical constituents. It contained 2.40-3.96% piperine (Li *et al.*, 1986). Das *et al.* (1996) identified the compounds from *P. longum* fruits. They were characterized from their spectral data as guineensine, piperine, N-isobutyl-2E, 4E-decadienamide, (+)-sesamin and 3-(3′,4′,5′-trimethoxyphenyl)-propanoic acid. Zhang *et al.* (1996) reported that four compounds were isolated from *P. longum* fruit. They are (z)-12-octadecenoic-α-glyceral monoester, piperine, β-sitosterol and daucosterol. Shankaracharya *et al.* (1997) studied on chemical compounds of long pepper. The result showed that long pepper contained about 1% volatile oil, 1.25% piperine and 40% starch. The GC-MS analyzed of the essential oil showed the presence of 48 components. The three major components of the oil were β-caryophyllene (17%), pentadecane (17.8%) and β-bisabolone (11.16%). Das, Kashinatham and Madhusadhan (1998) examined two samples from the fruits of *P. longum*. From one sample, a new alkamide, pergumidiene, and from other sample, two rare alkamides, brachystamide B and piperderdin were isolated. The known compounds, piperine, piperlonguminine, pellitorine, (+)-sesamine and 3-(3′,4′,5′-trimethoxyphenyl)-propanoic acid, were the common constituents of both two samples.

**Piper sarmentosum** Roxb.

**Synonym** : *Piper samentosum* Roxb.

**Family** : Piperaceae

**Name (Thai)** : Cha-phlu
Botanical descriptions:

*P. sarmentosum* Roxb. is a biennial; creeper follow ground, root to put forth at joint; have stand erect and tall 30-80 cm. Leaves on the lower surface densely or rather densely covered with patent short hairs on nerves and larger veins, lowest ones ovate-cordate, 5-7 nerved, 7-15 cm by 5-10 cm., petiole 2-8 cm; highest leaves very obliquely oblong, ± 3-nerved, rather long acuminate, 7-11 cm. by 3-5 cm.; petiole 1/3-1/2 cm.; midrib just above base with 1 ascending lateral nerve on either side. Flower male, female; spike erect to erect-patent, 1-2 cm.; peduncle 1/2-5/4 cm., patently short-hairy; bract ± circular, while, 3/4-1 cm. wide; stamens short; stigmas 3-4; berries connate and agnate to bract, with free opices, obovoid, dark green. Young twigs short-hairy (Backer, Bakhuizen and Brink, 1963).

Ethnomedical uses:

Entire plant: used as a stomachic, antipyretic
Fruits: dysentery,
Roots: dysentery

Pharmacological activities

The crude water extract of *P. sarmentosum* leaves has been demonstrated to reduce blood glucose in alloxan-induced diabetic rabbits, however, it has no effect in normal fasted rabbit. The administration of the extract to maturity-onset diabetic patients resulted in a reduction of blood glucose level. The methanolic extract from *P. sarmentosum* leaves, however, could not reduced blood glucose in normal rabbits (Pongmarutai, 1980). The hypoglycemic effect of the water extract of whole plant of *P. sarmentosum* was also examined in normal and streptozotocin-induced diabetic rats. The results showed that a single oral administration of the water extract at doses of 0.125 and 0.25 g/kg significantly lowered the plasma glucose level in the normal rats. The repeated oral administration of
the water extract at a dose of 0.125 g/kg for 7 days produced a significant hypoglycemic effect in the diabetic rats. (Peungvicha et al., 1998).

Eleven Thai medicinal plants were screened for their cathartics and antispasmodics activities in isolated rat ileum. It has been found that *P. sarmentosum* was able to decrease the intestinal tension and also inhibits the acetylcholine-induced intestinal tension (Apisariyakul and Anantasarn, 1984). In contrast, crude methanol extract of *P. sarmentosum* produced an increase in both frequency and amplitude of contraction of isolated guinea-pig ileum while atropine could only slightly antagonize the effect. The extract was also tested using rat phrenic nerve-hemidiaphragm preparation. The result showed that the extract produced a slightly twitch potentiation followed by twitch potentiation, then twitch depression. The authors concluded that the extract acted like a cholinergic compound on the ileum and depolarizing neuromuscular blocking agent on the neuromuscular junction. The latter activity was studied further using rat phrenic nerve-hemidiaphragm preparation. The authors concluded that the plant extract possessed a marked neuromuscular blocking activity at the neuromuscular junction. This is possibly due to an inhibition on the acetylcholine release at the presynaptic terminal (Ridtitid et al., 1998).

The essential oils, distilled from the leaves and fruits of *P. sarmentosum* inhibited the growth of *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Candida albicans* (Anpak et al., 1997).

The chloroform and methanolic extract of *P. sarmentosum*, *Andrographis paniculata* and *Tinospora crispa* were used to test for antimalarial effect *in vitro* and *in vivo*. The results showed that the chloroform extracts were more active than the methanolic extracts in *vitro*. Although the extract of *P. sarmentosum* was effective, the activity of *A. paniculata* demonstrated higher antimalarial activity than other two plants *in vivo* (Najib Nik et al., 1999).
Chemical constituents

Niamsa and Chantrapromma (1983) studied the chemical constituents of petroleum ether extract of *P. sarmentosum*. Two of them were identified as hydrocinnamic acid and β-sitosterol. Likhitwittayawud et al. (1987) also reported six components isolated from the fruit of *P. sarmentosum*. Two of them were the ubiquitous β-sitosterol and the known unsaturated amide. The other four components consisted of the aromatic alkene, the pyrole amide and two unsaturated pyridoline amides, which were name as sarmentine and sarmentosine. Masuda et al. (1991) identified the benzene soluble fraction of the methanolic leave extract of phenylpropanoids. They are 1-allyl-2,4,5-trimethoxybenzene, 1-(1-E-propenyl)-2,4,5-trimethoxybenzene, 1-allyl-2-methoxy-4,5-methylenedioxybenzene and 1-allyl-2,6-dimethoxy-3,4-methylenedioxybenzene. The same authors also showed that the compound, 1-allyl-2,4,5-trimethoxybenzene is asaron. The compound, 1-(1-E-propenyl)-2,4,5-trimethoxybenzene is α-asaron, which has been isolated from the fruit of the plant. Anpak et al. (1997) examined chemical compositions of essential oil distilled from the leaves and fruits of *P. sarmentosum*. This study revealed that longifolene (24.30%), β-caryophyllene (10.10%), allo-aromadendrene (13.51%) and 9-epi-(E)-caryophylene (18.24%) were the major constituents of the leaf oil whereas β-caryophyllene (31.11%), β-asaron (26.65%), viriflorene (9.28%) and β-selinene (8.21%) were the major constituents of the fruit oil.

*Quercus infectoria* Oliv.

Family: Fagaceae

Common name: Nut Gall, Aleppo Galls, White gall, Galls, Oak Galls

Name (Thai): Benganee
Botanical descriptions:

Trees, sometimes buttressed, rarely with small stilt-roots. Branchlets initially densely tomentose by simple or stellate hairs, or densely brownish, stiff pubescent, glabrescent; terminal buds ovoid-globous or ovoid-conical, rarely ovoid-ellipsoid, usually conflated, scales densely fulvous-tomentose, with a tendency to orthostichy. Stipples extrapetiolar, linear acute, densely tomentose or woolly pubescent, caduceus. Leaves spirally arranged, crowned near the top of the branchlets or rarely pseudo-whirled; midrib and nerves flattened or impressed or slightly raised above, prominent beneath; margin entire or remotely minutely serrate in the apical half; glabrous to densely punscent or tomentose at least on the lower surrace; petiole thickened at nase. Inflorescence male or female. Male rachis solitary in the axil of a lower leaf or in paniculate clusters on the lateral or subterminal new shoots, flexuous, pendent, compound or simple; bracts ovate-linear, acute, densely tomentose, caducous; male flowers in clusters of 3-4; perianth (4-) 6-lobed, the lobes connate at base, densely tomentose; stamens (4-) 6(-9), filaments slender, filiform, glabrous or tomentose at base, anther 0.5-1 mm long, basifixed; pistillode normally absent, sometimes replaced by a tuft of stiff simple hairs. Female rachis solitary in the axil of a higher leaf, erect, densely woolly pubescent, few- to many-flowered; bracts linear-acute, densely pubescent, caducous; female flowers always solitary; perianth (4-)6(-9)-lobed, staminodes 0 or 5-7, styles 3-4(-6), cylindrical, free and recurved or connate at base; stigmas broadly capitate, glabrous; ovary cells as many as styles. Cupule cup-or saucer-shaped, obconical or obovoid-globose, lamellate, hairy both inside and outside; lamellae c. 5-12, denticulate and free at the rim or more or less smooth and connate, thin or thick. Fruit ovoid-conical, ovoid-globose or ovoid-cylindrical; apex rounded, attenuate-acute or abruptly depressed, umbonte; perianthodium (umbo) provided with many rings, well-developed; glabrous and shining or densely tomentose.

Nut Gall is the excrescence obtained from the young twigs of *Quercus infectoria* Oliv. and allied species of *Quercus* (Fam. Fagaceae). The galls are obtained principally from Aleppo in Asiatic Turkey.

The excrescence (gall) is formed due to the puncture of a hymenopterous insect, *Cynips tinctoria*, which deposited ovum. The gall develops in several stages to the development of the insects as follows:

1. When the larva begins to develop and the gall enlarge, the cells of the outer and central zones contain numerous small starch grains.

2. When the chrysalis stage is reached, the starch near the middle of the gall is replace in part by gallic acid, but the peripheral and central cells contain masses of tannic acid.

3. As the winged insect is developed, nearly all of the cells contain masses of tannic acid with a slight amount of adhering gallic acid.

4. When the insect emerges from the gall, a hole to the central cavity is formed. Thus, the tannic acid, due to the presence of moisture and air, may be oxidized in part into an insoluble product, and the gall becomes more porous constituting the so-called White Gall of commerce.

**Ethnomedical uses**

Bark: diuretic, intestinal disinfectant, tiredness, backache and heart burn, hemorrhage, leucorrhrea, tuberculosis, rachitism, in alkaline or metal poisoning, in grand inflammation, petechia, dermatitis, eczema, impetigo, edema

Gall: digestant, hemorrhage, astringent

Leaf: astringent

Fruit: mild or severe diarrhea, indigestion, abdominal pain, anemia, rachitism, coughing, tuberculosis, at the onset of osteoporosis
Pharmacological activities

*Q. infectoria* nut gall has been studied for its pharmacological effects using various extraction methods. Intraperitoneal injection of crude methanolic extract of *Q. infectoria* was shown to be effective as an analgesic and a CNS depressant in rat and also showed hypoglycemic activity in female rabbit but with weak antiparkinson activity in mouse (Dar et al., 1976). A similar extract also inhibited alpha-glycosidases such as sucrase, maltase and isomaltase. This effect was comparable to acarbose, which is known to be a hypoglycemic agent (Hwang et al., 2000). A dried acetone-treated methanolic extract of the gall demonstrated an analgesic activity in rat and significantly reduced blood sugar levels in rabbit. A subfraction of this extract prepared by chloroform-methanolic extraction also had CNS depressant activity (Dar et al., 1976). Apart from its activities in animals, methanolic and water extract of *Q. infectoria* nut gall also showed an inhibitory effect on hepatitis C virus (HCV) protease in vitro (Hussein et al., 2000).

Chemical constituents

The identification of chemical constituents of *Q. infectoria* revealed that this plant contained ellagic acid, coumarin, betulinic acid methyl ester triterpene and steroid (Dar et al., 1976). It also contained tannin, galloyl-β-D-glucose, 1-2-3-4-6-penta-o, 1-2-3-6-tetra-o, 4-0-digalloyl-1-2-3-6-tetra-o, 6-o-digalloyl-1-2-3-4-tetra-o, 6-o-digalloyl-1-2-3-tri-o and 6-o-trigalloyl-1-2-3-tri-o (Nishizawa et al., 1983).

Gastrointestinal system

1. Structure of the gastrointestinal tract

The structure of the gastrointestinal tract is shown in Figure 4. The mucosa is the innermost layer of the gastrointestinal tract. It consists of an epithelium, the lamina propria, and the muscularis mucosae. The epithelium
is a single layer of specialized cell that lines the lumen of the gastrointestinal tract. The nature of the epithelium varies greatly from one part of the digestive tract to another. The lamina propria consists largely of loose connective tissue that contains collagen and elastic fibrils. The lamina propria is rich of several types of glands and contains lymph nodules and capillaries. The muscularis mucosae is the thin, innermost layer of intestinal smooth muscle. The mucosal folds and ridges are caused by contractions of the muscularis mucosae.

The next layer is the submucosa. The submucosa consists largely of loose connective tissue with collagen and elastin fibrils. Glands are present in the submucosa of some region of the gastrointestinal tract. The larger nerve trunks and blood vessels of the intestinal wall lie in the submucosa (Berne et al., 1998a).

The next layer, the muscularis externa, consists of two substantial layers of smooth muscle fibres, an outer layer with its fibres oriented in a longitudinal direction and an inner layer containing fibres with a circular orientation (Bray et al., 1999). The wall of the gastrointestinal tract contains many interconnected neurons (Figure 5 and 6). The submucosal plexus (Meissner's plexus) which lies between the circular muscles and the muscularis mucosae and the prominent myenteric plexus (Auerbach's plexus) which lies between the outer longitudinal and circular muscles layer (Bray et al., 1999). These intramural plexus, together with the other neurons of the gastrointestinal tract, constitute the enteric nervous system. These enteric nervous systems help to integrate the motor and secretory activities of the gastrointestinal system (Berne et al., 1998a). The serosa, or adventitia, is the outermost of the gastrointestinal tract. This layer consists mainly of connective tissue covered with a layer of squamous mesothelial cells (Berne et al., 1998a).
Figure 4  The general organization of the layers of the gastrointestinal tract  (Source: Berne et al., 1998a, p. 590)
2. Physiological function and its regulation

The major physiological functions of the gastrointestinal system are to digest foodstuffs and absorb nutrient molecules into the bloodstream. The gastrointestinal system carries out these functions by motility, secretion, digestion and absorption.

The function of the gastrointestinal tract are regulated and coordinated by hormones, paracrine agonists and neurons. The regulation may be classified as endocrine, paracrine, or neurocrine, depending on the cell type that produces the regulatory substance and the route of delivery of the substance to the target cell (Berne et al., 1998a).

This thesis concentrates on the effects of compounds on the intestinal motility. The following sections therefore focus mainly on the regulation of this function.

2.1 Gastrointestinal hormone

Endocrine cells are located in the mucosa or submucosa of the stomach and the intestine, as well as in the pancreas. These endocrine cells produce an array of hormones. Some of these hormones act on secretory cells located in the wall of the gastrointestinal tract, in the pancreas, or in the liver to alter the rate or the composition of their secretions. Other hormones act on smooth muscle cells in specific segments of the gastrointestinal tract, on gastrointestinal sphincters, or on the musculature of the gallbladder (Berne et al., 1998a). Some of the important hormones for motility control are as follows.

Cholecystokinin is secreted by I cell in the mucosal of the duodenum and jejunum mainly in response to the presence of breakdown products of fat, fatty acids and monoglycerides in the gastrointestinal contents. It has a potent effect in increasing contractility of the gallbladder, thus expelling bile into the small intestine where it emulsifies fatty substances allowing them to be digested and absorbed. Cholecystokinin also inhibits stomach motility moderately. Secretin is secreted by S cells in the mucosa of duodenum in
response to acidic gastric juice emptied from the stomach through the pylorus. It has a mild inhibitory effect on the motility of most of the gastrointestinal tract. Gastric inhibitory peptide is secreted by the mucosa of the upper small intestine, mainly in response to fatty acids and amino acids. It has a mild effect in decreasing motor activity of the stomach and therefore slows the emptying of gastric contents into the duodenum when the upper small intestine is already over supplied with food products (Guyton and Hall, 1996).

2.2 Paracrine mediators

Paracrine substances regulate the secretory and motor functions of the gastrointestinal tract. For example, histamine is released from cells in the wall of the stomach. The substance is a key physiological agonist of hydrochloric acid secretion by gastric parietal cells. Other paracrine agonists are released by cells of the extensive gastrointestinal immune system. The mass of cells with immune function in the gastrointestinal tract is approximately equal to the combined mass of immunocytes in the rest of the body. The gastrointestinal immune system secretes antibodies in response to specific food antigens and mounts an immunologic defense against many pathogenic microorganisms (Berne et al., 1998a).

The components of the gastrointestinal immune system include cells in mesenteric lymph nodes, Peyer’s patches in the wall of the intestine, and immunocytes that reside in the mucosa and submucosa. Mucosal and submucosal immunocytes include intraepithelial lymphocytes, B and T lymphocytes, plasma cells, mast cells, macrophages and eosinophils. These immune cells secrete inflammatory mediators such as histamine, prostaglandins, leukotrienes, cytokines and others. Once released, these mediators diffuse to secretory and smooth muscle cells in the gastrointestinal tract, where they affect their activities and modulate the function of neurons in the gastrointestinal tract. The gastrointestinal immune system is involved in some of the most trouble some
Figure 5  Neural control of the gut wall, showing (1) the myenteric and submucosal plexus; (2) extrinsic control of these plexuses by sympathetic and parasympathetic nervous system and (3) sensory fibres passing from the luminal epithelium and gut wall to the enteric plexuses and from these to the prevertebral ganglia, spinal cord, and brain stem. (Source: Guyton and Hall, 1996, p.796)
Figure 6  Diagram of the wall of the small intestine showing the two ganglionated plexuses: the submucosal and myenteric plexuses that comprise the enteric nervous system. Other structures that are shown include the lumen (L), the mucosa (f), the muscularis mucosa (g), the submucosa (e), the inner circular (i) and outer longitudinal (j) of the muscularis externa, and the serosa (k). Within the mesentery (c) are perivascular sympathetic input to the gut (b) and parasympathetic (vagal) fibres (a) entering the bowel in the mesentery. Other plexuses of nerve fibres that are indicated are mucosal nerves (d), the deep muscular plexus (h) between the circular muscle and submucosa. (Source: Gershon et al., 1994, p. 391).
gastrointestinal disorders, such as celiac disease and inflammatory bowel disease (Berne et al., 1998a).

2.3 Neural control

2.3.1 Innervations of the gastrointestinal tract (Figure 5 and 6)

2.3.1.1 Extrinsic innervation of the gut is through the autonomic nervous system, which is divided into parasympathetic and sympathetic components, and through sensory (afferent) nerves. The extrinsic nervous control involves the central nervous system and allows regulation and coordination of gastrointestinal activity over greater distances.

Afferent fibres run in association with the autonomic nerves supplying the gut. They carry information from chemoreceptors and mechanoreceptors located in the gut wall.

In the sympathetic nervous system, preganglionic cholinergic efferent fibres from the spinal cord (thoracolumbar segment) synapse with postganglionic adrenergic fibres outside of the gastrointestinal tract in prevertebral ganglia. Postganglionic fibres innervate the cells of myenteric and submucosal plexus (Johnson, 1992). Element from the enteric system then innervate smooth muscle, secretory and endocrine cells. Stimulation of sympathetic input to the gastrointestinal tract inhibits motor activity of the muscularis externa. However, this inhibitory effect of the sympathetic nerves does not result from direct action on the smooth muscle cells. Rather, the sympathetic nerves influence neural circuits in the enteric nervous system, these circuits provide input to the smooth muscle cells as previously mentioned (Berne et al., 1998a).

Parasympathetic innervation of the gastrointestinal tract down to the level of the transverse colon is provided by vagus nerve. The remainder of the colon, rectum and anus receive parasympathetic fibres from the pelvic nerve. These parasympathetic fibres are preganglionic and predominantly cholinergic. They arise from cell bodies within medulla (vagus) and the
sacral region of spinal cord (pelvic) (Johnson, 1992). The parasympathetic fibres terminate predominantly on the ganglion cells in the intramural plexuses. The ganglion cells then directly innervate the smooth muscle and secretory cells of the gastrointestinal tract. Excitation of parasympathetic nerves usually stimulates the motor and secretory activities of the gastrointestinal tract (Berne et al., 1998a).

In summary, although the enteric nervous system can function on its own, independently of these extrinsic nerves, the stimulating by the parasympathetic and sympathetic systems can further activate or inhibit gastrointestinal functions (Guyton and Hall, 1996).

2.3.1.2 Intrinsic innervation (enteric nervous system) is composed mainly of two plexuses: myenteric plexus or Auerbachs plexus situated between the muscle layers of the muscularis externa, and submucosal plexus or Meissner’s plexus that lies in submucosa (Figure 5 and 6). The myenteric plexus controls mainly the gastrointestinal movements, and the submucosal plexus controls mainly gastrointestinal secretion and local blood flow (Guyton and Hall, 1996).

The myenteric plexus consists of mostly linear chain of many interconnecting neurons or interneurons that extend the entire length of the gastrointestinal tract. Its function is concerned mainly with the control of motor activity along the length of the gut. When it is stimulated, its principal effects are (1) increased tonic contraction, or tone of the gut wall, (2) increased intensity of the rhythmic contractions, (3) slightly increased rate of the rhythm of contraction, and (4) increased velocity of contraction of excitatory waves along the gut wall, causing more rapid movement of the peristaltic waves. However, the myenteric plexus must not be considered entirely excitatory, some of the neurons are inhibitory. Besides motor neurons and interneurons, the myenteric plexus also contains sensory neurons (Guyton and Hall, 1996).
The intrinsic neurons which act directly on the smooth muscle of the gut wall are of two types. These are excitatory, mainly cholinergic neurons and inhibitory, nonadrenergic noncholinergic neurons. Excitatory motor neurons of myenteric ganglia release acetylcholine (ACh) on muscarinic receptors on the smooth muscle cells, they also release substance P. Inhibitory motor neurons release vasoactive intestinal polypeptide (VIP) and nitric oxide (NO). Most myenteric interneurons release ACh on to nicotinic receptors on motor neurons or on other interneurons (Berne et al., 1998a).

The submucosal plexus, in contrast to the myenteric plexus, is mainly concerned with controlling function within the inner wall of each minute segment of the intestine. For instance, many sensory signals originating from the gastrointestinal epithelium are integrated in the submucosal plexus to help control local intestinal secretion, local absorption, and local contraction of the submucosal muscle that causes various degrees of infolding of the stomach mucosa (Guyton and Hall, 1996).

Stimulatory secretomotor neurons release ACh and VIP onto gland cells or epithelial cell. Submucosal interneurons release ACh onto other neurons in submucosal ganglia or project to myenteric ganglia. The submucosal ganglia also have numerous sensory neurons, which are the afferent limbs of the secretomotor reflexes. Most of the sensory neurons respond to chemical stimuli or mechanical deformation of mucosa (Berne et al., 1998a).

2.3.2 Types of neurotransmitters secreted by the enteric neurons

In an attempt to a better understanding of the multiple functions of the enteric nervous system, research workers over the world have identified a dozen or more of different neurotransmitters that are released by the nerve ending of different type of enteric neurons. Two of them with which we are already familiar are (1) acetylcholine and (2) norepinephrine. Others are (3) adenosine triphosphate, (4) serotonin, (5) dopamine, (6) cholecystokinin, (7) substance P, (8) vasoactive intestinal polypeptide, (9)
somatostatin, (10) leu-enkephalin, (11) met-enkephalin, and (12) bombesin. The specific functions of most of these neurotransmitters are not well understood enough to justify extensive discussion, other than to point out the following: Acetylcholine most often excites gastrointestinal activity. Norepinephrine, on the other hand, almost always inhibits gastrointestinal activity. This is also true of epinephrine, which reaches the gastrointestinal tract by way of the blood after it is secreted by the adrenal medulla into the circulation. The other transmitter substances listed above are a mixture of excitatory and inhibitory agents, but their importance and even their functions are still mainly to be determined (Guyton and Hall, 1996). Details of the neurotransmitters including their locations and roles are summarized in Table 1.

3. Contraction of intestinal smooth muscle

Contraction of smooth muscle is regulated by the cytosolic Ca\(^{2+}\) level, and the sensitivity to Ca\(^{2+}\) of the contractile elements in response to change in the environment surrounding the cell (Karaki et al., 1997).

Calcium plays an important role in physiology and pathophysiology of numerous cell types, including the gut smooth muscle cells and the enteric neurons. In the smooth muscle cells, an increase in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) is an essential step for the cells to contract. The increase in [Ca\(^{2+}\)]\(_i\) occurs by influx from the extracellular medium and by Ca\(^{2+}\) release from the rapidly exchanging intracellular stores (Makhlouf, 1991). Ca\(^{2+}\) influx occurs through ion channels in the plasmalemma. The opening of these channels in smooth muscle cells is regulated by membrane depolarization (voltage-operated channels, VOCS or voltage-gated channels, VGCS) or by chemical gating of receptor operated channels activation (receptor-operated channels, ROCs, or ligand-gated channels) (Meldolesi and Pozzan, 1987; Nathan, 2003).
3.1 Ca\textsuperscript{2+} influx through plasmalemma calcium channels (Figure 7)

3.1.1 Voltage-operated calcium channels (VOCs)

VOCs is opened by depolarization or during action potential membrane depolarization (Bolton et al., 1999). VOC are classified into six subtypes: L-, N-, P-, Q-, R- and T-types. In smooth muscle, only the L-type Ca\textsuperscript{2+} channel is considered to be a major Ca\textsuperscript{2+} influx pathway (reviewed by Karaki et al., 1997). L-type Ca\textsuperscript{2+} channels have been found in all regions of the gastrointestinal tract and they are required for gastrointestinal smooth muscle contractility. Addition of nifedipine, an L-type Ca\textsuperscript{2+} channel blocker, to intestinal smooth muscle strip results in cessation of the contractility (reviewed by Farrugia, 1999). It has long been known that high potassium solution can depolarized plasma membrane and opened calcium channel (Bolton, 1979). In intestinal smooth muscle, as in other varieties of smooth muscle, exposure to potassium-rich solution elicit contractions that are dependent on extracellular calcium (reviewed by Godfraind et al., 1986). These channels can also be regulated by receptor-mediated and intracellular messenger. Agonists can open L-type calcium channels by depolarizing the cell membrane through activation of the nonselective cation channels, inhibition of the K\textsuperscript{+} channel and/or activation of the Cl\textsuperscript{-} channel (Figure 8; Carl et al., 1995 and reviewed by Karaki et al., 1997). Some agonists may open the L-type Ca\textsuperscript{2+} channels directly as for Bay K8644 and endogenous Ca\textsuperscript{2+}, and indirectly through GTP-binding proteins in the absence of membrane depolarization (Figure 9; van Breeman, 1989; reviewed by Karaki et al., 1997).

3.1.2 Receptor-operated calcium channels (ROC) are stimulated by many ligands including neurotransmitters, hormones, cytokines and antigen rather than (without) changes in membrane potential. Receptor activation may open ROCs directly or indirectly through GTP-binding protein and second messengers which may be inositol 1, 4, 5-trisphosphate (IP\textsubscript{3}),
Inositol 1, 3, 4 5-tetrakisphosphate (IP₄) or Ca²⁺ (Figure 9; van Breeman, 1989; Karaki et al., 1997). The plasmalemma also contains channels that are controlled indirectly by the state of fullness of the sarcoplasmic reticulum (SR) Ca²⁺ stores, through a mechanism known as capacitative Ca²⁺ entry. In this scheme, the SR Ca²⁺ stores behave like a capacitor in that they inhibit Ca²⁺ entry when they are fully loaded, but as they discharge they begin to open calcium release activated Ca²⁺ (CRAC) channels. The mechanism by which Ca²⁺ stores modulate CRACs in the plasmalemma is unclear. One suggestion is that the empty Ca²⁺ stores release a messenger, calcium influx factor (CIF), which diffuses to the membrane and opens the CRACs (Berridge and Bootman, 1997).

### 3.2 Ca²⁺ release from internal stores (Fig. 7 and 10)

As mentioned above, the source of cytosolic free Ca²⁺ are both extracellular and intracellular. The SR is the physiological intracellular source and sink of intracellular Ca²⁺ in smooth muscle (Somlyo and Somlyo, 1994). There are two families of intracellular channels responsible for releasing Ca²⁺ from the intracellular store: inositol 1,4,5-trisphosphate (InsP₃Rs or IP₃Rs) and ryanodine receptors (RyRs) (Figure 10; Berridge and Bootman, 1997). In intestinal smooth muscle, there are about 10 times more IP₃Rs than RyRs (Bolton et al., 1999).

#### 3.2.1 IP₃ receptor/calcium release channels

When many G protein-link receptors are activated, IP₃ is formed in increased amount through stimulation of phospholipase C activity and activation of phosphatidyl inositol-4,5-bisphosphate (van Breeman, 1989 and Bolton et al., 1999).

IP₃ receptor consists of three domains: an IP₃-binding amino-terminal domain, a regulatory domain containing ATP-binding and phosphorylation sites and a carboxy terminal domain containing six transmembrane regions. The transmembrane regions are responsible for the aggregation of four
subunits into the functional tetrameric receptor protein, and also serve to form the Ca\textsuperscript{2+} channel. The four identical subunits, each contains an IP\textsubscript{3}-binding site in the large N-terminal cytosolic domain. The binding of IP\textsubscript{3} to the amino-terminal induces a conformation change in the protein. (Berridge and Bootman, 1997).

The opening of calcium channel in response to IP\textsubscript{3} is potentiated by low (below 300 nM) and inhibited by high concentration (above 300 nM) of calcium (Karaki et al., 1997 and Bolton et al., 1999). Therefore, cytoplasmic calcium and IP\textsubscript{3} synergize in opening the IP\textsubscript{3}-receptor channels. ATP also potentiates the opening of the channel (Bolton et al., 1999). The action of IP\textsubscript{3} on its receptor is blocked by heparin, which does not block and may potentiate the opening of RyRs (Bolton et al., 1999).

3.2.2 Ryanodine receptor / calcium release channels

The ryanodine receptor (RyR) is a release channel that become activated when the cytosolic Ca\textsuperscript{2+} rises to produce the phenomenon of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) (Figure 7 and 10; Missiaen et al., 1992). RyR is first identified in muscle cells owing to their strong affinity for the plant alkaloid, ryanodine extracted from Ryania speciosa (Berridge and Bootman, 1997). Two types of RyR function are believed to exist: in skeletal muscle, a direct coupling of membrane depolarization to RyR channel opening is about by an association between a voltage- and dihydropyridine-sensitive calcium channel and the RyR; in cardiac muscle, a process of CICR occurs whereby calcium entering through these calcium channels trigger further calcium release through RyR from the SR stores. At present, there are evidences suggested that the smooth muscle RyR resembles that in cardiac muscle, there is no direct coupling as seen in skeletal muscle (reviewed by Bolton et al., 1999). RyR is activated by Ca\textsuperscript{2+}, cyclic ADP ribose (Figure 10) and caffeine and is blocked by ryanodine alkaloid. It has been suggested
<table>
<thead>
<tr>
<th>Substance</th>
<th>Location and role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine (ACh)</td>
<td>Primary excitatory transmitter to muscle, to intestinal epithelium</td>
</tr>
<tr>
<td>Adenosine triphosphate (ATP)</td>
<td>Probably contributes to transmission from enteric inhibitory muscle motor neurons</td>
</tr>
<tr>
<td>Calcitonin gene-related peptide (CGRP)</td>
<td>Present in some secretomotor neuron and interneurons; role unknown</td>
</tr>
<tr>
<td>Cholecystokinin (CCK)</td>
<td>Present in some secretomotor neuron and in some interneurons; may contribute to excitatory transmission; generally excites muscle</td>
</tr>
<tr>
<td>Dynorphin (DYN) and dynorphin-related peptides</td>
<td>Present in secretomotor neuron, interneurons and motor neurons to muscle; does not appear to be a primary transmitter</td>
</tr>
<tr>
<td>Enkephalin (ENK) and enkephalin-related peptides</td>
<td>Present in interneurons and muscle motor neurons</td>
</tr>
<tr>
<td>Galanin</td>
<td>In most regions these substances probably provide feedback inhibition of transmitter release</td>
</tr>
<tr>
<td>Gastrin-releasing peptide (GRP) (mammalian bombesin)</td>
<td>Present in secretomotor neurons, descending interneurons and inhibitory motor neurons in human intestine; role unknown</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>Excitatory transmitter to gastrin cells</td>
</tr>
<tr>
<td>Nitric oxide (NO)</td>
<td>Also found in nerve fibres to muscle and in interneurons, where its roles are not unknown</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>Present in secretomotor neurons, where it appears to inhibit secretion of water and electrolytes</td>
</tr>
<tr>
<td></td>
<td>A cotransmitter from enteric inhibitory muscle motor neurons</td>
</tr>
<tr>
<td></td>
<td>Noradrenergic nerve fibres in the intestine are not strictly enteric: they are of sympathetic</td>
</tr>
</tbody>
</table>
Table 1  Substances that may be neurotransmitters or neuromodulators in the enteric nervous system (continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Location and role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotonin (5-HT)</td>
<td>origin\nMajor roles are to inhibit motility in nonsphincter regions, to contract the muscle of the sphincters, to inhibit secretomotor reflexes and to act as vasoconstrictor neurons to enteric arterioles</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Appears to participate in excitatory neuroneuronal transmission\nDespite its widespread distribution in enteric neurons, no clearly defined roles have been established</td>
</tr>
<tr>
<td>Tachykinin (substance P, neurokinin A, neuropeptide K and neuropeptide γ)</td>
<td>Excitatory transmitters to muscle; and are cotransmitters with ACh\nMay contribute to excitatory neuroneuronal transmission</td>
</tr>
<tr>
<td>Vasoactive intestinal peptide (VIP) (and peptide histidine isoleucine [PHI])</td>
<td>Excitatory transmitter from secretomotor neurons\nPossibly a transmitter of enteric vasodilator neurons\nContributes to transmission from enteric inhibitory muscle motor neurons</td>
</tr>
</tbody>
</table>

Reference: Berne et al. (1998a)
Figure 7 1. Sources of intracellular Ca\textsuperscript{2+}:
   a. from extracellular, Ca\textsuperscript{2+} enters through voltage-gated and ligand-gated Ca channels;
   b. from intracellular store, Ca\textsuperscript{2+} is released from sarcoplasmic reticulum (SR) by a rise in cytosolic Ca\textsuperscript{2+} (Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release) or by inositol triphosphate (IP\textsubscript{3}), a second messenger, binding to its receptor.

2. Removal of excess intracellular Ca\textsuperscript{2+}:
   a. by Ca-ATPase (Ca pump) in the SR membrane;
   b. by Na-Ca exchanger in the plasmalemma;
   c. by Ca-ATPase (Ca pump) in the plasmalemma.

(Source: Nathan, 2003 http://www.ttuhs.edu/SOM/physiology/courses/HumanPhysiology/ Nathan/HumanPhysiologyMuscle.pdf)
Figure 8  Positive and negative-feedback loops regulating Ca\(^{2+}\) entry. 

\(K^{+}_{\text{dr}}\), Delayed rectifier \(K^{+}\) channels; \(X^{+}\), Ca\(^{2+}\)-facilitated nonselective cation channels; \(Cl^{-}\), Ca\(^{2+}\)-activated \(Cl^{-}\) channels; Ca\(^{2+}\), voltage-dependent Ca\(^{2+}\) channel; \(K^{+}_{\text{ca}}\), Ca\(^{2+}\)-activated \(K^{+}\) channels. Effects of Ca\(^{2+}\) on \(K^{+}_{\text{dr}}, X^{+}\), and \(Cl^{-}\) tend to decrease membrane potential (depolarize) and increase excitability (V\(\downarrow\)). This enhances Ca\(^{2+}\) entry via voltage-dependent Ca\(^{2+}\) channels and generate positive feedback. Activation of \(K^{+}_{\text{ca}}\) and inactivation of Ca\(^{2+}\) channels tend to increase membrane potential (hyperpolarize) and/or decrease excitability (V\(\uparrow\)). These pathways constitute negative feedback. Major Ca\(^{2+}\) sources include entry through Ca\(^{2+}\) channels and release of Ca\(^{2+}\) from sarcoplasmic reticulum (SR), and either source can contribute to regulation of ion channels in the plasma membrane. (Source: Carl et al., 1995, p.C10).
Figure 9  Putative mechanisms for activation of $\text{Ca}^{2+}$ entry by agonists. Receptor activation may open receptor-operated $\text{Ca}^{2+}$ channels (ROCs) directly or indirectly via the second messengers $\alpha$, inositol 1,4,5-triphosphate (IP$_3$), inositol 1,3,4,5-tetrakisphosphate (IP$_4$) or $\text{Ca}^{2+}$. Agonists may also open voltage-gated $\text{Ca}^{2+}$ channels (VGCs) directly, as for Bay K8664 and endogenous $\text{Ca}^{2+}$ agonists (ECA), and indirectly through activation of protein kinase C by diacylglycerol (DAG). The latter process inhibits G-protein activation and $[\text{Ca}^{2+}]$, has been shown to inactivate VGCs. (Source: van Breeman, 1989, p.319).
that [Ca\textsuperscript{2+}], must exceed 1 μM for CICR to occur (Berridge and Bootman, 1997; reviewed by Bolton et al., 1999). There are evidences suggest that the smooth muscle RyR channel probably behave similarly to those from cardiac and skeletal muscles, whereby ryanodine and its analogs cause the channel to open and then be fixed in a partial open state or, at higher concentrations, to be blocked (reviewed by Bolton et al., 1999). In guinea-pig tenia ceacum, ryanodine stimulated the rate of leak of the Ca\textsuperscript{2+} store, so that the drug behaved as a functional Ca\textsuperscript{2+} store blocker through the depletion of Ca\textsuperscript{2+} in the store.

Although the RyR shares feature with the IP\textsubscript{3}R, such as a homotetrameric structure, it is not activated by IP\textsubscript{3} (Horowitz et al., 1996). There is finding support that the ryanodine and inositone IP3- sensitive stores constitute physically distinct pools of readily releasable calcium and these two distinct intracellular calcium pools coexist in the smooth muscle from the longitudinal and circular layers (Oh et al. 1997).

3.3 Calcium Off mechanism

The increase in cytosolic Ca\textsuperscript{2+} is usually transient. Once the ‘On mechanism’ have generated a Ca\textsuperscript{2+} signal by introducing Ca\textsuperscript{2+} into the cytoplasm, the OFF mechanism begin the process of recovery by returning Ca\textsuperscript{2+} either to the stores or back to the external medium. These recovery pathways have to be extremely active because they need to remove not only the free cytosolic Ca\textsuperscript{2+}, but also the 100–fold large amount that is bound to Ca\textsuperscript{2+} buffer or calcium binding protein such as parvalbumin (Berridge and Bootman, 1997). The cytosolic Ca\textsuperscript{2+} can be reduced, causing muscle relaxation, though several mechanism carried out by pump and exchangers (Figure 7 and 11).

1) There are two basic mechanisms by which calcium is removed by cells. The first mechanism involves an ATP-dependent Ca\textsuperscript{2+} pump or plasmalemma Ca\textsuperscript{2+}-ATPases (PMCA)s which utilize the energy of
Figure 10 Calcium-mobilizing intracellular messengers.

1. **Inositol 1,4,5 triphosphate (InsP₃).** This messenger is formed from the membrane phospholipid phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂], which is cleaved in response to hormonal stimulation is cleaved in response to hormonal stimulation to give diacylglycerol (DAG) and InsP₃. InsP₃ diffuses into the cytoplasm, binds to its receptor and release store Ca²⁺.

2. **Calcium.** The most important diffusable messenger for activating InsP₃R and ryanodine receptors (RyR). The process of Ca²⁺-induced Ca²⁺ release (CICR) is of fundamental important in regulating the way in which cell mobilize Ca²⁺ from internal stores. Trigger Ca²⁺ can either enter from outside through plasmalemma channels [Voltage-operated channels (VOC), Receptor-operated channels (ROC) or Calcium-release activated Ca²⁺ channels (CRAC)] or it can be released from intracellular stores. Ca²⁺ can act as the sole trigger for activating RyRs, however InsP₃Rs usually require the simultaneous presence of Ca²⁺ and InsP₃.

3. **Cyclic adenosine diphosphate ribose (cADPR).** This messenger is formed from the cellular metabolite NAD⁺ and potently activates Ca²⁺ release from RyR. (Source: Berridge and Bootman, 1997, p. 205).
ATP to transport Ca\(^{2+}\) against the enormous electrochemical gradient that exists. The second mechanism is the sodium-calcium (Na\(^{+}/Ca\(^{2+}\)) exchanger. The exact mechanism, by which this exchanger works, is unclear. It is known that Ca\(^{2+}\) and Na\(^{+}\) can move in either direction across the plasma membrane. The direction of movement of these ions (either inward or outward) will depend upon the membrane potential and chemical gradient for the ions. Furthermore, three Na\(^{+}\) ions are exchanged for each Ca\(^{2+}\). However, Ca\(^{2+}\)-ATPases plays a more important role in Ca\(^{2+}\) extrusion than does Na\(^{+}/Ca\(^{2+}\) exchange (Karaki et al., 1997, Berridge and Bootman, 1997).

2) The sarcoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCA) pump are located in the membrane of intracellular stores, where they function to sequester the Ca\(^{2+}\) introduced into cytoplasm (Berridge and Bootman, 1997). The rate and extent of Ca\(^{2+}\) pumping into sarcoplasmic reticulum (SR) is sufficient to cause relaxation (reviewed by Somlyo and Somlyo, 1994). The SERCA are inhibited by thapsigargin and cyclopiazonic acid (Karaki et al., 1997).

3.4 Mechanism of smooth muscle contraction

The intracellular free calcium level ([Ca\(^{2+}\)]\(_i\)) in smooth muscle is a major determinant of smooth muscle contractility (Figure 7, 11 and 12) (Horowitz et al., 1996, Nathan 2003 and Faber, 2003). The development of force results from MgATP dependent cyclic interactions of myosin in thick filaments with actin in thin filaments. The force of contraction, in turn, is regulated by the concentration of free Ca\(^{2+}\) surrounding these myofilaments (Kamm and Stull, 1989).

The consequences of increase in ([Ca\(^{2+}\)]\(_i\)) levels in the smooth muscle cell is the binding of Ca\(^{2+}\) to calmodulin (CAM) and the resulting Ca\(^{2+}\)-calmodulin complex then bind to myosin light chain kinase (MLCK) and activate it. The MLCK catalyzes the phosphorylation of the 20kDa myosin light chain (LC\(_{20}\)) on serine at position 19. The phosphorylation allows the
actin/myosin adenosine triphosphatase (ATPase) to be activated, and actin slides on myosin, producing contraction.

A fall in cytosolic \( \text{Ca}^{2+} \) concentration, by being pumped out of the cell or pumped into the SR stores, inactivates MLCK and permit dephosphorylation of LC\(_{20}\) phosphatase, thus deactivating the myosin ATPase and causing relaxation (Somlyo and Somlyo, 1994, Lodish et al., 1999; Ganong, 2001).

### 3.5 Changes in calcium sensitivity

As mentioned above, in smooth muscle, \( \text{Ca}^{2+}\)-calmodulin regulate myosin-light-chain kinase (MLCK) which phosphorylates myosin light chains, enabling myosin to interact with actin and thereby initiating contraction. Relaxation is usually initiated by a fall in \([\text{Ca}^{2+}]\), which leads to dephosphorylation of the myosin light chain via myosin phosphatase. The relatively indirect coupling between \([\text{Ca}^{2+}]\), and contraction in smooth muscle allows for contraction to be regulated by mechanisms that increase or reduce the sensitivity of the contractile apparatus to \([\text{Ca}^{2+}]\), as follows:

- decrease activity of myosin phosphatase or increase activity of MLCK results in \( \text{Ca}^{2+} \) sensitization;
- increase activity of myosin phosphatase or decrease activity of MLCK causes \( \text{Ca}^{2+} \) desensititation (Rang et al., 1999).

#### 3.5.1 Increase in calcium sensitivity

There are evidences showing that the use of \( \text{Ca}^{2+} \) indicators to reveal the force/ \( \text{Ca}^{2+} \) ratio is variable, and generally higher during activation by agonist than by a depolarizing-induced increase in \([\text{Ca}^{2+}]\), indicating a \( \text{Ca}^{2+} \)-sensitizing effect of agonists. Agonists (for example, \( \alpha_1 \)-adrenergic and muscarinic) can also increase force in permeabilized smooth muscle in which \([\text{Ca}^{2+}]\), is clamped with chelators and intracellular \( \text{Ca}^{2+} \) compartmentalization and cytoplasmic \( \text{Ca}^{2+} \) gradients. Conversely, force
Figure 11  Mechanisms of smooth muscle contraction and signal transduction mechanisms. G, heterotrimeric GTP binding protein; PLC, phospholipase C; PIP₂, Phosphatidylinositol 4,5 bisphosphate; PC, phosphatidylcholine; IP₃, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; SR, sarcoplasmic reticulum; MLCK, myosin light chain kinase; PKC, protein kinase C; CaD, caldesmon; CaP, calponin; MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; Gₓ, small GTP-binding protein; CaBP, calcium-binding protein; CR, calreticulum; CSQ, calsequestrin; PA, phosphatidic acid; CaM, calmodulin; RyR, ryanodine receptor; LC₂₀, 20-kDa myosin light chain; CaMKII, Ca²⁺/calmodulin protein kinase II. Dashed lines indicate pathways that may require kinases or cofactors not yet defined. (Source: Horowitz et al., 1996, p. 968)
Figure 12  Mechanisms of agonists-induced contraction and relaxation.
can decline while cytoplasmic Ca\(^{2+}\) is maintained, indicating a decrease in Ca\(^{2+}\)-sensitivity. Such increases and decreases in force at constant Ca\(^{2+}\) are known to result from parallel changes in the activities of phosphorylating and dephosphorylating enzymes and, consequently, in LC\(_{20}\) phosphorylation (reviewed by Somlyo and Somlyo et al., 1994).

Inhibition of protein phosphatase is the main and perhaps only mechanism of G-protein-coupled Ca\(^{2+}\)-sensitization. Inhibition of the phosphatase increases LC\(_{20}\) phosphorylation and hence the level of force at a given [Ca\(^{2+}\)]. It is proposed that agonists increase the Ca\(^{2+}\)-sensitivity of contractile elements by activating a GTP-binding protein since the sensitizing effect can be inhibited by GDP-βS, a competitive inhibitor of GTP, and mimicked by GTP-γS, a GTP analogue resistant to hydrolysis (reviewed by Somlyo and Somlyo et al., 1994; Karaki et al., 1997).

Both protein kinase C and arachinodic acid can inhibit myosin phosphatase. Protein kinase C is activated by diacylglycerol (DAG), the hydrolysis product of phosphatidylinositol biphosphate and of phosphatidylcholine by phospholipase C. Phospholipase C is activated by agonist G-protein complex. The main evidence for a role of protein kinase C is the Ca\(^{2+}\)-sensitizing effect of phorbol esters, which are well known activator of protein kinase C. There are reports showing that phorbol ester decreases the rate of relaxation and LC\(_{20}\) dephosphorylation suggesting protein kinase C increase Ca\(^{2+}\)-sensitivity through the inhibition of myosin phosphatase (Itoh et al., 1993, Masuo et al., 1994; Horowitz et al., 1996). The agonist G-protein complex can also activate phospholipase A\(_2\), which in turn, cleaves arachidonic acid from membrane phospholipid. It has also been reported that arachidonic acid release is associated with inhibition of dephosphorylation of myosin light chain in intact smooth muscle tissue (Gong et al., 1995).
3.5.2 Decrease in calcium sensitivity

Phosphorylation of a specific serine residue in the region of the calmodulin-binding domain of myosin light-chain kinase (site A of MLCK) by several kinases reduces its affinity for calcium calmodulin and consequently its phosphorylating activity. Phosphorylation of MLCK by one of these kinases, calmodulin kinase II (CAMKII), occurs in vivo, and is a physiological mechanism of desensitization, although it requires a higher cytoplasmic [Ca$^{2+}$], than does activation of MLCK, owing to the lower affinity of CAMKII compared with that of MLCK for Ca$^{2+}$-calmodulin (reviewed by Somlyo and Somlyo, 1994).

The increases in cyclic AMP due to β-adrenergic stimulation and in cyclic GMP due to nitric oxide result in inhibition of contraction in intact smooth muscle. Simultaneous measurements of [Ca$^{2+}$], and muscle force, however, showed that these cyclic nucleotide more strongly inhibited contraction than [Ca$^{2+}$], suggesting that cyclic nucleotides caused muscle relaxation by desensitization of contractile elements to Ca$^{2+}$ (reviewed by Karaki et al., 1997).

4. Agonist-induced muscle contraction

4.1 Acetylcholine

4.1.1 Acetylcholine (ACh) is the principal excitatory neurotransmitter of the parasympathetic nervous system, being released from both ganglionic synapses and at postganglionic neuro-effector junctions. The release of ACh can also induced by distension of the gut and consequent activation of stretch receptor. This local reflex causes excitation of cholinergic interneurons and thus modulates the direction and magnitude of peristaltic activity.

4.1.2 ACh contracts visceral smooth muscle through the stimulation of muscarinic receptors, of which five subtypes are known (M$_1$-M$_5$). Intestinal smooth muscle expressed predominantly M$_2$ and M$_3$ subtypes, the
proportion of which has been estimated to be 70-80% and 20-30%, respectively. However, pharmacological studies have determined that M₃ receptors and to a lesser extent M₂ receptors, are involved in intestinal muscle contraction (Eglen, 2001). The role of muscarinic receptors in intestinal smooth muscle contraction has been re-examined through the use of knockout mice. In M₃ receptor knockout mice, carbachol-induced contraction in the ileum, gall bladders and stomach fundus were reduced by about 75%, with the residual response displaying a pharmacological profile consistent with M₂-mediated contraction (reviewed by Lecci et al., 2002).

4.1.3 The stimulation of M₃ receptor is link to activation of membrane bound enzyme phospholipase C (PLC) via the pertussis toxin insensitive G₄ protein. Agonist activation of the M₃ receptor therefore increase PLC activity and accelerates the rate of phosphatidylinositol 4,5-biphosphate (PIP₂) hydrolysis. The breakdown of PIP₂ leads to the formation of inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) (Caulfield and Birdsall, 1998), and these breakdown products acts as second messengers by mobilizing Ca²⁺ from intracellular stores and activating protein kinase C, respectively (Nahorski et al., 1997 and Lecci et al., 2002). It is generally assumed that generation of IP₃ leads to a rapid phase of muscle contraction, because the peak of IP₃ generation precedes the initiation of contraction. The phosphorylation of several contractile proteins by various isoforms to PKC in smooth muscle plays a key role in muscle contraction (Caulfield and Birdsall, 1998). M₂ receptors are coupled to a pertussis toxin sensitive G protein to inhibit adenyl cyclase activity, a mechanism that is able to switch off relaxatory signals mediated by an increased synthesis of cAMP in responses to β-adrenoceptor agonist (Figure 13; Eglen et al., 1994, Nahorski et al., 1997 and Lecci et al., 2002). In addition, there are evidences showing that the activation of muscarinic M₂ receptors decreases the opening times of a potassium channel activated by β-adrenoceptor agonist, also attenuating relaxation induced by sympathetic system (Kotlikoff et al., 1999). By these
process, therefore, it appears that the muscarinic arm of the parasympathetic system serves not only to contract smooth muscle directly (via activation of muscarinic M3 receptors) but also to abrogate sympathetically mediated relaxation (via activation of M2 receptors)

4.1.4 Muscarinic M2 receptors also open a nonselective cation channel, thereby augmenting entry of extracellular sodium ions (reviewed by Bolton et al., 1999). As these channel have a low permeability to calcium ion, induction of this muscarinic M2 receptor-mediated cationic current elicits membrane depolarization and subsequent entry of calcium ion via voltage selective L-type channels (Eglen, 2001). Cationic current (Ica) is very sensitive to increases in the free calcium concentration on the inner side of the membrane (Inou and Isenberg, 1990; Pacaud and Bolton, 1991). Bolton and Zholas (1997) showed that the cationic current evoked by carbachol which normally cause depolarization of muscle is inhibited competitively by M2 receptor antagonist. However, M3 antagonist strongly reduced the maximum cationic current which could be evoked by carbachol in a noncompetitive manner. Since [Ca²⁺], rises when M3 receptors are activated due to PLC activation, IP₃ formation and calcium store release. A rise in [Ca²⁺], strongly potentiate the cationic current, M3 receptors may, by this mechanism, effectively control the size of cationic current gated by M2 receptor activation. Blocking M3 receptors may seriously attenuate the M2-cationic link. Thus, cation channel are gated by M2 receptor activation but strongly modulated by activation of M3 receptors.

4.2 Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) has a major role in the regulation of gastrointestinal motility. About 90% of the body 5-HT is synthesized and stored by enterochromaffin cells in the mucosa, and 10% is present in the enteric neurons. It is released from enterochromaffin cells as a paracrine agent or from enteric neurons as a neurotransmitter in the gastrointestinal tract. Basal release of enteric 5-HT is augmented by
mechanical stretching, such as that caused by food or the administration of hypertonic saline, and also by efferent vagal stimulation. 5-HT causes contraction of gastrointestinal smooth muscle, increasing tone and facilitating peristalsis. This action is partly due to the direct action of 5-HT on 5-HT$_{2A}$ receptors on smooth muscle and partly due to an indirect excitatory action on 5-HT$_{4}$ receptors on enteric neurons (Katzung, 2001; Sanders-Bush and Mayer, 2001).

The 5-HT$_{2A}$ subtype activates a G protein, termed $G_{q}$, that is responsible for stimulation of phospholipase C activity; the immediate result is hydrolysis of phosphatidylinositol biphosphates (which are components of the plasma membrane) to form inositol-1,4,5-triphosphates. The inositol triphosphate causes release of intracellular Ca$^{2+}$ from stores in the endoplasmic reticulum. Thus, these receptors mediate such Ca$^{2+}$-dependent phenomena as contraction of smooth muscle and secretion. The second product of the phospholipase C reaction, diacylglycerol, activates protein kinase C in modulation of function and in the later phase of functional response (Hoyer et al., 1994).

4.3 Histamine

Histamine released in response to many stimuli including urticaria, inflammation and diarrhea. Histamine-releasing substances can activate the secretory response of mast cells or basophils by causing a rise in intracellular Ca$^{2+}$.

Histamine-induced intestinal smooth muscle contraction appears to be a pure H$_{1}$-receptor mediated effect (Leurs et al., 1991). As in the responses to ACh and 5-HT, the primary mechanism by which histamine H$_{1}$-receptors produce functional response in cells is the activation of phospholipase C via a pertussis toxin-insensitive G-protein, probably $G_{q/11}$. This causes the generation of IP$_{3}$ which inducing the intracellular calcium mobilization and muscle contraction (Hill et al., 1997 and Brown and Robert, II, 2001).
Figure 13 The balance between the relaxant and contractile state of smooth muscle depends on the prevailing parasympathetic and sympathetic drive. In this model both muscarinic M₂ and M₃ receptors modulate the contractile state of the tissue. M₃ receptors mediate contraction, by coupling to the G protein G₉, mobilizing inositol (1,4,5)-triphosphate (IP₃) and diacylglycerol (DAG) and, consequently, elevating intracellular Ca²⁺ levels. Stimulation of β-adrenoceptors causes relaxation by enhancement adenyl cyclase activity while activation of M₂ receptors by coupling to Gᵢ inhibits this augmentation (Source: Eglen et al., 1994, p.118).
4.4 Differences in signal transductions during the initial phases of contraction between circular and longitudinal muscle

Although it is generally known that the contraction of intestinal smooth muscle by various agonists are due to the increase in cytosolic free Ca\(^{2+}\) released from intracellular store activated by IP\(_3\), as mentioned above. However, there are evidences showing that the mechanism mediating the initial transient phase of contraction in both circular and longitudinal muscles are different (Makhlouf and Garbriel, 1997). In circular muscle, the source of Ca\(^{2+}\) responsible for agonist-induced contraction is intracellular sarcoplasmic reticulum mediated by IP\(_3\). The selective increase of IP\(_3\) in circular muscle cells followed a pattern of preferential hydrolysis of PIP\(_2\) observed with various agonists (e.g. CCK-8, ACh, 5-HT and histamine) in different species (Grider and Makhlouf, 1988, Murthy and Makhlouf, 1995, Morini et al., 1993; Kuemmerle et al., 1995). The initial Ca\(^{2+}\) transient in circular muscle cells is not affected by Ca\(^{2+}\) channel blocker or withdrawal of Ca\(_{2+}\) from the medium (Grider and Makhlouf, 1988; Murthy et al., 1991). Depletion of Ca\(^{2+}\) stores with the sarcoplasmic Ca\(^{2+}\)/ATPase inhibitor, thapsigargin, abolish the agonist-induced increase in [Ca\(^{2+}\)], confirming the initial step in Ca\(^{2+}\) mobilization is the release of Ca\(^{2+}\) from sarcoplasmic store (Kuemmerle et al., 1995).

However, Ca\(^{2+}\) release in intestinal longitudinal muscle exhibit an obligatory dependence on an initial step involving Ca\(^{2+}\) influx into the cell. The agonist-induced increase in [Ca\(^{2+}\)], Ca\(^{2+}\) release from sarcoplasmic stores and initial contraction are abolished in Ca\(^{2+}\)-free medium or in the presence of Ca\(^{2+}\) channel blocker (Grider and Makhlouf, 1988 and Murthy et al., 1991 and Kuemmerle et al., 1995). The Ca\(^{2+}\) mobilization in this muscle involves a cascade initiated by agonist-induced transient activation of phospholipase A\(_2\) (PLA\(_2\)) by pertussis toxin-sensitive (PTX) G-protein and formation of arachinodic acid (AA), AA-depolarization of the plasma membrane and opening of voltage-sensitive Ca\(^{2+}\) channels. The influx of
Ca\textsuperscript{2+}-induces Ca\textsuperscript{2+} release by activating sarcoplasmic ryanodine receptor/Ca\textsuperscript{2+} channels and stimulates cADPR formation which enhances Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (Makhlof and Murthy, 1997). The effect of AA is not exerted directly on plasmalemma Ca\textsuperscript{2+} channels but involves activation of Cl\textsuperscript{-} channels resulting in depolarization of and opening of voltage-sensitive Ca\textsuperscript{2+} channels (Kuemmerle, Murthy and Makhlof, 1994). However, others presented evidences that the agonist-induced membrane depolarization were due to the opening of nonselective cation channels (Inou and Isenberg, 1990, Pacaud and Bolton, 1991; Komori et al., 1992) or the suppressing of Ca\textsuperscript{2+}-activated K\textsuperscript{+} current (Figure 12; Sim et al., 1985; Cole et al., 1989; Carl et al., 1995 and Faber, 2003).

4.5 Signal transduction during sustained smooth muscle contraction

As previously mentioned, the initial [Ca\textsuperscript{2+}], transient in both circular and longitudinal muscle cell types result in Ca\textsuperscript{2+}/calmodulin-dependent activation of MLC kinase, phosphorylation of MLC\textsubscript{20} and interaction of actin and myosin. In contrast, the sustained phase of contraction is mediated by a Ca\textsuperscript{2+}-independent isoform of PKC, PKC\textepsilon (Makhlof and Murthy, 1997).

The pattern of PKC activation parallels that of DAG formation, with a sustained phase coinciding with that of sustained muscle contraction. The mechanism whereby PKC regulate sustained contraction has not been defined. It is possible that PKC regulate the MLC-dependent slowly cycling crossbridges. Alternatively, actin-binding proteins (e.g., caldesmon and/or calponin) may be involved that are initiating interaction of actin and myosin (Figure 11; Horowitz et al., 1996 and reviewed by Makhlof and Murthy, 1997).

Although Makhlof and Murthy (1997) presented evidences that only the initial transient contraction was Ca\textsuperscript{2+}-dependent but the sustained phase was mediated by Ca\textsuperscript{2+}-independent pathway, there are some works
demonstrated that both phases of contraction were Ca\(^{2+}\)-dependent (Brading and Snedden, 1980; Morel et al., 1987; Sato et al., 1994; Dessy and Godfraind, 1996). Sato et al. (1994) showed that both the initial and sustained phases of ACh (1\(\mu\)M)-induced-contraction of canine colonic circular muscle were abolished or reduced by nicardipine (1\(\mu\)M) or verapamil (10 \(\mu\)M), while the increase in [Ca\(^{2+}\)] was reduced by about 30%. Thus, they suggested that the influx through voltage-dependent Ca\(^{2+}\) channels and release of Ca\(^{2+}\) from intracellular stores contribute to the regulation of [Ca\(^{2+}\)], by ACh. Dessy and Godfrained (1996) also reported that the phasic and tonic contractions and the increase in [Ca\(^{2+}\)], produced by histamine (10 \(\mu\)M), in guinea-pig ileum longitudinal muscle were abolished by the L-type calcium channel blockers, nimodipine (1 \(\mu\)M) and D600 (10 \(\mu\)M).

4.6 Signal transduction mechanisms mediating smooth muscle relaxation

Relaxant agents (e.g. nitric oxide, vasoactive intestinal polypeptide, isoproterenol) act mainly by stimulating the formation of cAMP and/or cGMP, resulting in activation of cAMP-dependent protein kinase A (PKA) and/or cGMP-dependent protein kinase G (PKG) (reviewed by Makhlof and Murthy, 1997). Both PKA and PKG inhibit the initial [Ca\(^{2+}\)], and decrease the sensitivity of MLC kinase to [Ca\(^{2+}\)], (Miller et al., 1988; Murthy et al., 1993; Murthy and Makhlof, 1995). The decrease in [Ca\(^{2+}\)], in gastric and intestinal circular smooth muscle involves inhibition both IP\(_3\) formation and IP\(_3\)-dependent Ca\(^{2+}\) release (Murthy et al., 1993). Stimulation of Ca\(^{2+}\) uptake into the sarcoplasmic reticulum attenuates [Ca\(^{2+}\)], but this mechanism appears to be subsidiary and confined to PKG. Inhibition of voltage-sensitive Ca\(^{2+}\) channels by PKA or PKG reduces Ca\(^{2+}\) influx, as does stimulation of K\(^+\) channels which leads to hyperpolarization of the plasma membrane and inactivation of Ca\(^{2+}\) channels (Kume et al., 1989; McDaniel et
\( al., 1992; \) Chen and Rembold, 1992); (see Figure 15) (Makhlouf and Murthy, 1997) and Figure 12 (Faber, 2003). Inhibition of PLA\(_2\) activity and AA formation in longitudinal muscle and inhibition of Ca\(^{2+}\) or cADPR-induced Ca\(^{2+}\) release in longitudinal muscle cells have not been studied (Makhlouf and Murthy, 1997).

4.7 Cross-signaling in gastrointestinal smooth muscle

A single agonist can interact with multiple receptors coupled to distinct G-proteins and/or effector enzymes, thereby initiating interacting cascades (see Figure 13 and 14; Eglen et al., 1994; Makhlouf and Murthy, 1997).

5. Potassium chloride-induced intestinal contraction

There are reports that high potassium containing solution causes a biphasic contraction, an initial rapid, transient phasic component which is followed by a slowly developing and prolonged tonic component in guinea-pig ileum and guinea-pig taenia coli. High potassium produces a smooth muscle contraction by causing membrane depolarization, opening the L-type Ca\(^{2+}\) channel and increase in Ca\(^{2+}\) influx. Thus, contraction induced by high K\(^+\) is considered to be due to a relatively simple mechanism, an increase in [Ca\(^{2+}\)], without changing other signal transduction systems including phosphatidylinositol turnover and Ca\(^{2+}\) sensitization (Karaki et al., 1997).

6. Loperamide and antidiarrheal activity

Loperamide is a widely used opiate antidiarrheal agent that acts predominantly on \( \mu \) opioid receptors in the gastrointestinal tract (Awouters et al., 1993; Brunton et al., 1996). It is a piperidine opioid that is 40-50 times more potent than morphine as an antidiarrheal agent. It penetrate the CNS very poorly. Thus, loperamide lacks significant abuse potential and preferred to other agents (Brunton, 1996 and Jafri and Paricha, 2001). It has been reported that at oral antidiarrheal doses, about 80% of the administered loperamide is confined to the gastrointestinal tract and the
enterohepatic circulation. Normal visceral barriers provide low levels of loperamide in the systemic circulation (≤ 0.3% of the dose at time peak levels) and are the first limit to penetrate into the brain (Heykants et al., 1974). Within the intestinal wall, loperamide concentrates in the longitudinal muscle-myenteric plexus layer (van Nueten et al., 1979) and low concentrations of [³H]loperamide label villus tips of rat and human mucosa, apparently on μ and δ opioid receptors (reviewed by Awouters et al., 1993). As with other opioids, the antidiarrheal effect of loperamide is due to the antimotility and antisecretory activities mediated principally by opioids receptors on enteric nerves, epithelial cells and muscle (Brown, 1995; Jafri and Paricha, 2001). Loperamide decreases peristalsis or propulsive contraction and increasing nonpropulsive type segmentation contraction. The effect results from its action at μ-opioid receptors on myenteric plexus to decrease acetylcholine release and accordingly reduce propulsive movement of the longitudinal smooth muscle. The increase in segmentation contraction is due to the inhibition of the release neurotransmitters (VIP, nitric oxide) from myenteric neurons innervating the circular smooth muscle (Brown, 1995). Moderate to high dose (≥12 mg) of loperamide delay small intestinal and whole gut transit in human. The delay in transit leads to increase net uptake of fluid by the mucosa as a result of increase in contact time (Awouters et al., 1993). Furthermore, loperamide also exert important effects at μ- (or δ-) opioid receptors on submucosal neurons that lead to a net increase in salt and water absorption, and this effect are reversed by naloxone (Brown, 1995).

Some actions of loperamide have been found to be not or only partially naloxone reversible and are therefore presumably not mediated by μ-opioid receptors. The effects may be δ-opioid receptors activating effects or non-opioiod effects (Awouters et al., 1993). The non-opioid effects of loperamide include functional inhibition of calmodulin which contributed to its antisecretory effect (Zaveczi et al., 1982; Diener et al., 1988 and Stoll et al.,
1988) and the inhibition of Ca\textsuperscript{2+}-dependent contraction and secretion
(Kenakin et al., 1982; Chang et al., 1984; Reynold et al., 1984; and Honda et al., 1994). In addition, Yagasaki et al. (1978) demonstrated inhibition of acetylcholine and prostaglandin release from guinea-pig ileum by loperamide, an effect that could not be reversed by naloxone. In Ca\textsuperscript{2+}-free solution, loperamide and verapamil had no influence of PGE\textsubscript{1}-induced contraction, but markedly inhibited Ca\textsuperscript{2+}-induced contraction (Honda et al., 1994).
**Figure 14** Signaling cascade initiated by contractile agonist in intestinal longitudinal muscle cells. The cascade represents events during the initial phase of contraction. Activation of phospholipase $A_2$ with formation of arachidonic acid (AA) is mediated by the $\beta\gamma$ subunits of an inhibitory G protein. AA activates Cl$^-$ channels resulting in depolarization of the plasma membrane and opening of voltage sensitive Ca$^{2+}$ channels. Ca$^{2+}$ influx induces Ca$^{2+}$ release by activating sarcoplasmic ryanodine receptor/Ca$^{2+}$ channels. The increase in [Ca$^{2+}$], activates membrane-bound ADP ribosyl cyclase. The resultant formation of cyclic adenosine diphosphate ribose (cADPR) enhance Ca$^{2+}$-induced Ca$^{2+}$ release. (Source: Makhlouf and Murthy, 1997, p. 271)
Figure 15  Dual signaling cascade initiated by interaction of VIP with two receptor subtypes. The VIP-preferring receptors are recognized by the homologous peptides, PHI (Peptide Histidine Isoleucine) and PACAP (Pituitary Adenyl Cyclase-Activating Polypeptide): these receptors are coupled via a PTx-sensitive G protein (G_i) to stimulation of Ca^{2+} influx and activation of a constitutive isoform of nitric oxide synthase (eNOS) which is bound to calmodulin (CAM) in the plasma membrane. The resultant generation of NO leads sequentially to activation of a soluble guanylyl cyclase (GC) and cGMP-dependent protein kinase (PKG), PKG is also cross-activated at high concentrations of agonists. PKA and PKG are jointly responsible for smooth muscle relaxation and hyperpolarization of the plasma membrane. (Source: Makhlouf and Murthy, 1997: 274)