CHAPTER 5

DISCUSSION AND CONCLUSION

The aim of this study is to determine the effect and classify mechanism of papaya leaves alkaloid (PA) on the isolated rat uterus. The crude alkaloid extract (9.1 gm) used throughout this thesis was prepared by one extraction. The yield of crude alkaloid extract was approximately 0.414% of the dried leaves weight. Carpaine have been reported to be a major alkaloid of *Caricar papaya* (Gupta et al., 1990) and found in all green parts of the plants. The yield of carpaine is ranging from 0.0115 – 0.4% (Burdick, 1971; Gupta et al., 1990) depending on the region where this plant grows (Morton et al., 1977). Apart from carpaine, pseudocarpaine, nicotine and unidentified alkaloid have been found in papaya leaves (Gupta et al., 1990); however, the yield of these alkaloid extract have not been published. Due to the fact that the alkaloid extract used in this study is crude in nature, it might contain all of above mentioned alkaloids. One of the alkaloids and other substance contained in the extract might be an active constituent that produced the effect in this study. On the other hand, some of them might work in concert to cause the effect. This issue has yet to be determined.

Our preliminary finding showed that the PA had no effect on uterine contraction in isolated rat uterus. However, PA was able to completely inhibit uterine contraction precontracted by depolarizing solution (high KCl solution) and uterine stimulants such as oxytocin, acetylcholine and $PGF_{2\alpha}$.

This findings suggested that the PA is a uterine relaxant. The result from this study is consistent to a study reviewed by Burdick (1971) that the carpaine (an alkaloid of *Carica papaya*) is a uterine relaxant.

One major finding of this study was the demonstration of the inhibitory effect of PA on the uterine contraction precontracted by depolarizing solution or induced by $CaCl_2$. PA and verapamil inhibited uterine contraction precontracted with depolarizing solution in a dose dependent manner and completely inhibited this contraction at the highest dose used. PA or verapamil also inhibited uterine contraction induced by $CaCl_2$ in Ca^{2+}-free-high KCl (60 mM) solution. Both mechanisms of depolarizing solution and $CaCl_2$ induced uterine contraction in Ca^{2+}-free high KCl solution are contributed to voltage-dependent Ca^{2+} channel. In depolarizing solution, the high KCl concentration (56.3 mM) depolarized the cell membrane, change the ions conductance of the cells which turned to activate voltage-dependent Ca^{2+} channel leading to Ca^{2+} influx inside the cells and then stimulated uterine contraction. This contraction is inhibited by Ca^{2+} channel antagonist such as verapamil (Revuelta et al., 1997) which is consistent to the effect of verapamil in this study. Similarly, in Ca^{2+}-free-high KCl solution, KCl also changed ions conductance which turned to activate voltage-dependent channel to opened stage (Vagha, 1998). Although Ca^{2+} channel was opened, the lack of Ca^{2+} in Ca^{2+}-free solution could not stimulate the uterus to contract. The addition of $CaCl_2$ to the organ bath caused the Ca^{2+} to enter the cell via opened Ca^{2+} channel and stimulated the uterine contraction. This study has found that PA completely inhibited uterine contraction induced by depolarizing solution and

also inhibited $CaCl_2$-induced contraction in Ca^{2+}-free-high KCl solution. It is suggested that the effect of PA may be related to a reduction in Ca^{2+} influx. In addition, the inhibitory effect of PA on $CaCl_2$-induced uterine contraction in Ca^{2+}-free-high KCl solution is similar to the effect of verapamil, a calcium channel antagonist (Vaghy, 1998). This result suggested that the inhibitory effect of PA on Ca^{2+} influx may occur through voltage-dependent Ca^{2+}-channel in the same manner as verapamil.

Oxytocin and $PGF_{2\alpha}$ induced the isolated rat uterus to contract rhythmically in Jalon-Ringer solution. The force of contraction produced by these stimulants was quite stable throughout the experiment. Although the frequency of contraction declined slowly with time, it has never been less than 80% of the initial frequency at the end of the experiment. In such a case, statistical analysis was performed using ANOVA to assure if the reduction of frequency of contraction was due to the drug treatment or the time-effect. In contrast to oxytocin and $PGF_{2\alpha}$, the force and frequency of uterine contraction induced by ACh were irregular and both parameters seemed to decline with time. Carbachol, a long-acting cholinergic agonist, has also been tested and produced a similar effect to ACh (data not shown). It seems likely that this phenomenon is not due to a rapid destruction of ACh by the enzyme cholinesterese. Due to this difficulty dealing with ACh, an experimental design using cumulative concentration of uterine relaxant in the organ bath can not be used properly. We, therefore, decided to use an experimental design which the uterus was preincubated with a single concentration of a uterine relaxant following with a stimulation by ACh.

It is well established that the uterus contains various specific receptors for many classes of uterine stimulants and uterine relaxants. Oxytocin, $PGF_{2\alpha}$ and ACh are known to be natural-occurring uterine stimulants. The interaction of these stimulants on their own receptors on uterus can induce uterine contraction. At present, there are no known agonists or antagonists that can cross-react with these 3 types of receptors. PA has been shown to inhibit uterine contraction caused by the 3 agents as well as depolarizing solution and $CaCl_2$. It seems unlikely that PA could produce the effect by interfering the binding of those stimulants to their receptors.

The action of oxytocin, $PGF_{2\alpha}$ and ACh which induce the uterus to contract is due to an increase in intracellular Ca^{2+} resulting from both Ca^{2+}-influx via plasma membrane and Ca^{2+}-release from internal stores (Monga and Sanborn, 1992). The latter action of oxytocin, $PGF_{2\alpha}$ and ACh which induced Ca^{2+}-release from internal storage are meditated by IP_3 signaling pathway. It is well known that all receptors of oxytocin, $PGF_{2\alpha}$ and ACh are G-protein-coupled receptors. The interaction between these agonists to their receptors on plasma membrane leading to an activation of G-protein coupled to PLC, hydrolysis of PIP_2 to IP_3 and then IP_3 will activate its receptor at SR resulting in Ca^{2+}-release from internal storage. The evidence has shown that the action of oxytocin is mediated through the hydrolysis of PIP_2 to IP_3 to induce Ca^{2+} release. However, the contractile effect of oxytocin is markedly inhibited by substance within a group of Ca^{2+} channel blocker. It is suggested that the major action of oxytocin to induce uterine contraction is mediated by an increase in Ca^{2+}-influx from extracellular space whereas the action that

mediated Ca^{2+}-release from internal stores plays a minor role (Marc et al., 1988; Rall, 1991). In an addition, the action of ACh is mediated by IP_3 to induce Ca^{2+} release, however, extracellular Ca^{2+} plays more importance role for the contraction induced by ACh (Reviewed by Wray, 1993; Monga and Sanborn, 1992). Molnar and Hertelendy, (1990) showed that oxytocin and $PGF_{2\alpha}$ triggered an increase in intracellular Ca^{2+} by different mechanisms. Oxytocin acts to activate G-protein-dependent PLC resulting to induce Ca^{2+}-release from internal stores, whereas the action of $PGF_{2\alpha}$ depends on extracellular Ca^{2+}. However, Fu et al., (2000) showed that $PGF_{2\alpha}$ triggered an increase intracellular Ca^{2+} by both cytosolic Ca^{2+} release from IP_3 sensitive stores and influx from extracellular space (Phillippe et al., 1997; Fu et al., 2000). It is therefore concluded that the actions of oxytocin, $PGF_{2\alpha}$ and ACh to induce an increase in intracellular Ca^{2+} are mediated by both Ca^{2+}-influx via plasma membrane and Ca^{2+}-release from internal stores but the major source of Ca^{2+} is the influx via plasma membrane. The results of this study showed that PA was able to inhibit contractile effect of oxytocin, $PGF_{2\alpha}$ and ACh. Verapamil also had a similar effect. It is suggested that the inhibition pathway of PA may occur through an inhibition on Ca^{2+}-influx via plasma membrane. However, its effect on the Ca^{2+} release from internal storage can not be excluded.

It is well established that agonists such as oxytocin, ACh or $PGF_{2\alpha}$ trigger Ca^{2+} influx to the cell via plasma membrane by two type of Ca^{2+} channel, one channel is voltage-dependent channel and the other is receptor-operated channel (Bolton, 1979; McDonal et al., 1994). Voltage-dependent

Ca^{2+} channels are selectively inhibited by organic Ca^{2+} channel blocker such as verapamil, whereas receptor-operated channel are not sensitive to Ca^{2+} channel antagonist or less markedly inhibited than voltage-dependent Ca^{2+} channel (Bolton, 1979). This was evidenced by an experiment to show that muscle contraction induced by agonist markedly inhibited by Ca^{2+} channel antagonist (McDonal, et al., 1994). This would happen in such a way that, the agonists bind to its receptor at the plasma membrane, activates receptor-linked channel to open and allows cations such as Na^{+} or Ca^{2+} to enter the cell, and/or generate signal pathway which induces Ca^{2+} release from internal storage. An increase of Ca^{2+} or the other cations in cytosol results a change in membrane potential which turns to activate voltage-dependent Ca^{2+} channel to open, increases Ca^{2+} influx into the cell leading to muscle contraction. Thus, the substance within group of Ca^{2+} channel blocker is able to inhibit this contraction because it inhibit the effect on Ca^{2+} influx via voltage-dependent Ca^{2+} channel (Bolton, 1979; Karaki et al., 1997). This study found that uterine contraction induced by oxytocin and acetylcholine was inhibited by verpamil. It is suggested that ions channel which contributed to the effect of oxytocin, ACh or $PGF_{2\alpha}$, at least in part, is the voltage-dependent channel. Therefore, the inhibitory pathway of PA on uterine contraction induced by oxytocin, ACh and $PGF_{2\alpha}$ which reduce Ca^{2+} influx may be due to an inhibition on Ca^{2+} influx via voltage-dependent Ca^{2+} channel similar to the action of verapamil.

PA and verapamil has shown to inhibit rhythmic contraction induced by oxytocin and $PGF_{2\alpha}$ in the isolated rat uterus on both frequency and force of contraction. The effect of PA or verapamil on the frequency of contraction

indicated that PA or verapamil might somehow affect the action potential generating system on the uterine muscle cells. It is known that the basal membrane potential undergoes rhythmic electrical changes called slow wave. At the threshold potential, there is a fast depolarization that generate an action potential on top of the slow wave. The action potential is attributed primarily to entry of Ca^{2+} (and Na^{+} in late pregnancy), through voltage sensitive Ca^{2+} channels (and also through fast Na^{+} channel in late pregnancy) (Monga and Sanborn, 1992; Sanborn, 2000). At the end of action potential the membrane repolarized, which is thought to involve the opening of K^{+} channels. Shortly after the repolarization, the depolarized action processes begin again and a new action potential occurs. This cycle continues again and again causing rhythmic excitation of tissue (Guyton, 1986; Karaki et al., 1997). Oxytocin, $PGF_{2\alpha}$ and ACh are known to stimulate Ca^{2+} influx across the plasma membrane. However, the action of these stimulants on action potential is not fully understood. It is known that action potential occurs in uterine muscle cells by an increase in permeability of plasma membrane to Na^{+} or Ca^{2+}. If any substrate acts to decrease the permeability of Na^{+} or Ca^{2+}, the depolarized phase of action potential could be largely delayed resulting in a decrease in frequency of contraction. In addition, an increase in the permeability of plasma membrane to K^{+} that causes K^{+} efflux contributes to repolarization phase of action potential. The next depolarization phase of action potential can not happen if K^{+} channel is still activated (Mehta et al., 1995; Anwer et al., 1993). If substrate acts to promote opened stage of K^{+} channel, it may delay the action potential leading to a reduction of the frequency of contraction. From

this point of view, it is therefore suggested that PA may act to decrease plasma membrane permeability to Ca^{2+} or Na^{+}, or to increase the permeability of membrane to K^{+}. This would cause a delay of the action potential and then, slower the frequency of contraction.

The contractility of the uterus is contributed to the rise of Ca^{2+} which is due to influx via plasma membrane and release from internal storage. In order to determine of the action of PA which is related to intracellular process, oxytocin (10 mU/ml) was used to induced uterine contraction in Ca^{2+}-free solution. This experiment was performed to exclude the effect of oxytocin which was related to Ca^{2+} influx. The result of this study showed that oxytocin (10 mU/ml) stimulated a phasic contraction which was quite stable throughout the experiment. However, the amplitude of contraction is only 1-5% of the contraction obtained from a similar stimulation in Ca^{2+}-containing solution. This contraction was similar to those reported by Oishi et al., (1991). PA was able to inhibit this contraction induced by oxytocin in Ca^{2+}-free solution. This result suggested that PA may diffuse across the plasma membrane and interact to some intracellular processes which involve in the uterine contraction resulting in uterine relaxation.

As already discussed, the intracellular mechanism of oxytocin is contributed to both Ca^{2+}-dependent and Ca^{2+}-independent pathway. The intracellular pathway of oxytocin, in term of Ca^{2+}-independent, may be related to as follows: 1) Activating PLC, hydrolyzed PIP_2 to IP_3 which induces Ca^{2+} release from IP_3 sensitive stores (Edward et al., 1986 and Rall, 1991). 2) Inhibiting Ca^{2+} extrusion at sarcolemma by inhibit Ca^{2+}-ATPase.

3) Inhibiting SR Ca^{2+}-ATPase which pump Ca^{2+} in cytosol to be stored at internal storage or 4) inhibiting Ca^{2+}-Na^{+} exchanger. It is therefore suggested that PA may affect one or more of the following intracellular processes. 1) The inhibition of IP_3-induced Ca^{2+} release, 2) The activation of Ca^{2+}-ATPase pump at sarcolemma which pump Ca^{2+} in cytosol to extracellular, 3) the activation of SR Ca^{2+}-ATPase which pump Ca^{2+} in cytosol to refill at internal storage or 4) the inhibition of Na^{+}-Ca^{2+} exchanger. However, the precise mechanisms of PA on intracellular process have yet to be identified. Furthermore, verapamil (10^{-5}-10^{-4} M) which used to compare the effect with PA partially inhibits this contraction. This effect of verpamil suggested that, at higher concentrations, verapamil may also affect the intracellular process.

For the intracellular Ca^{2+}-dependent pathway, the contraction of uterine muscle may occur as a result of a phosphorylation on either contractile of cytosolic protein leading to an increase in Ca^{2+} sensitivity of contractile apparatus (reviewed by Wray, 1993: Oishi et al., 1991). As mentioned above, PA may diffuse across plasma membrane to interact with and inhibit some intracellular processes that control the muscle contraction. Possible sites of action of PA were proposed in Figure 53. The rise in intracellular Ca^{2+} caused an increase in the binding of Ca^{2+} to CaM to form Ca^{2+}-CaM complex. The Ca^{2+}-CaM complexes are able to activate $MLCK^{P}$ (inactive from) to be an active MLCK. The active MLCK then phosphorylates inactive MLC which activates this protein (MLC^{P}). The activated MLC then interacts with actin and causes muscle contraction. In addition, the inactive MLCK ($MLCK^{P}$) can be dephosphorylated by various phosphatase enzyme which also results in the

active form (MLCK). The active MLCK, in turn, is inactivated by Ca^{2+} - calmodulin-dependent protein kinase II (CaMKII) (Karaki et al., 1997). From this point of view, PA that diffuses across the plasma membrane might inhibit phosphatase enzyme like calyculin, a protein phosphatase inhibitor, (Yuan et al., 1997) or activates CaMKII like bradykinin or ionomysin, a CaMK II activator, (Sironi et al., 1998; Fan et al., 1997). Moreover, the active MLC (MLC^P) which further interacts with actin leading to contraction is inactivated by MLC phosphatase (PPase). The active PPase is further change to inactive PPase by a tyrosine kinase or C kinase. However, only an active form of PPase is able to converted active MLC (MLC^P) to an inactive MLC (MLC). From a similar view as above, PA might act in such a way to decrease Ca^{2+} sensitivity of contractile apparatus by activate PPase enzyme like octreotide, a phosphatase activator (Yamashita et al., 1999), or activates tyrosine kinase like diperoxvanadate, a tyrosine kinase activator (Garcia et al., 1999) or C kinase like Phorbol-12-dibutyrate, a protein kinase C activator, (Shi et al., 2002) which rapidly turned an active MLC (MLC^P) to an inactive form (MLC). Furthermore, if PA prevent the binding of Ca^{2+} to CaM like trifluoperazine or W7, a calmodulin inhibitor, (Zhu et al., 2000; Ozaki et al., 1987), the amount of Ca-CaM complexes available for the initiation of contraction will be reduced. Thus these could be the action of PA that might work to reduce Ca^{2+} sensitivity of the contractile system. These mechanisms have to be identified.

It is well known that various agonists activate their receptors to generate IP_3 and DAG (reviewed by Horowitz et al., 1996). The function of IP_3 contributes to the induction of Ca^{2+} release from internal storage, as already

discussed above. For the function of DAG, many researchers have shown that it is related to the activation of protein kinase C (PKC). They also showed that PKC activation is important to control the component on sustained phase of agonist induced contraction in smooth muscle (reviewed by Horowitz et al., 1996). The pathway of PKC which involves in muscle contraction is still not clear. However, it may contributed to an increase in $[Ca^{2+}]_i$ (reviewed by McDonald, et al., 1994), or cause an increase in Ca^{2+} sensitivity of contractile apparatus (reviewed by Horowitz et al., 1996) by phosphorylation of other protein kinase, or involves in receptor-ions pump and or ion channel (reviewed by McDonald et al., 1994). From this point of view, if PA is able to diffuse across the plasma membrane to interact at intracellular site, PA might inhibit PKC by inhibiting DAG generation, or PA might directly inhibit PKC leading to a decrease in the contractile response of the uterus. The effects of PA on PKC or DAG have to be identified.

It is well established that the β-adrenergic receptors in the myometrium are mainly of β_2-subtype and the activation of $\beta_2$receptors causes uterine relaxation (Lands et al., 1967). The present study has shown that nonspecific β-adrenergic receptor agonist, isoproterenol (10^{-10}-10^{-5} M), produced concentration-dependent relaxation in KCl-depolarized uterus, and this effect was abolished by pretreatment with a β-adrenergic receptor antagonist propanolol (10^{-7} M) (Ehlert, 1995). This result is consistent with the known classical interaction of β-adrenergic agonist and antagonist on the uterine

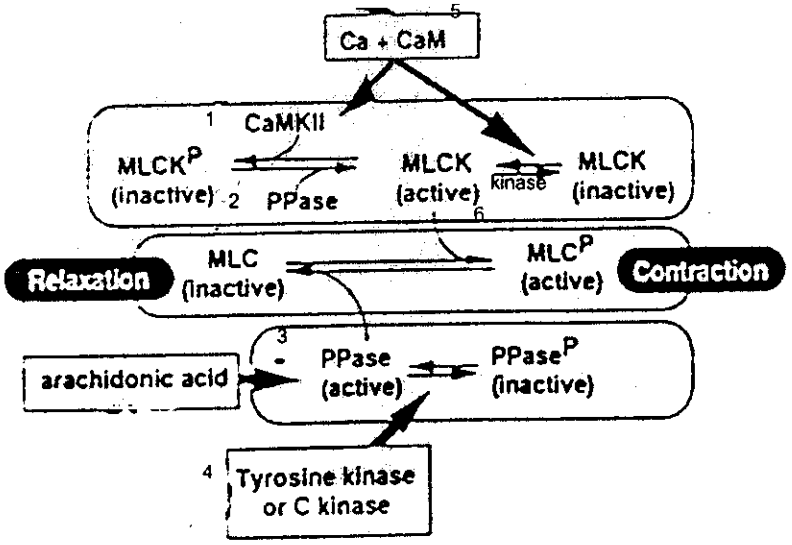

Figure 53 . Schematic representation of the possible sites of action of PA on contractile apparatus. 1) Activation of Ca^{2+}-calmodulin dependent protein kinaseII. 2) inhibition of phosphatase protein. 3) Stimulation of MLC phpsphatase. 4) Inhibition of C kinase and tyrosine kinase. 5) Preventation the binding of Ca^{2+} to calmodulin. 6) Activation of protein kinase. (Source: modified from Karaki et al., 1997, pp. 179).

relaxation reported elsewhere (Perez-Guerrero et al., 1996). PA alone caused a relaxation of the KCl-depolarized uterus similar to the effect of isoproterenol. Although propranolol (10^{-7} M) was able to inhibit the effect of isoproterenol, it had no effect on the relaxing effect of PA. This result suggested that the relaxing effect on uterine contraction of PA may be unrelated to the interaction with β_2-adrenergic receptor.

β_2-adrenergic receptor is known to couple to adenylate cyclase via intermediate G protein (Gs) The interaction of β-adrenergic agonist on this receptor causes the stimulation of adenylate cyclase which accelerates the conversion of ATP to cAMP. The rise in intracellular cAMP then activates cAMP-dependent kinase (protein kinase) which, in turn, phosphorylates proteins and causes uterine relaxation (see reviewed by Wray, 1993). The termination of the effect occurred by the hydrolysis of cAMP. This reaction is catalyzed by several phosphodiesterase (PDE) which convert cAMP to 5'AMP (Ross, 1999). The inhibition of this enzyme by PDE inhibitor such as papavarine may cause an accumulation of intracellular cAMP which further leads to uterine relaxation (Revuelta et al., 2000). Although the effect of PA is not like to be mediated via β_2-adrenergic receptor activation, a PDE inhibition may be the site of action of PA.

In summary, the result of this study indicated that PA is a uterine relaxant. The relaxing effect of PA is unlikely to be mediated by an activation of β_2-adrenergaic receptor. The inhibitory effect of of PA may involve an inhibition of a rise in intracellular Ca^{2+} from both Ca^{2+} influx via plasma membrane and Ca^{2+} release from internal storage. However, this effect may be mediated mainly through a reduction on Ca^{2+} influx, possibly through voltage-dependent Ca^{2+} channel. Possible intracellular mechanisms of PA which may contribute to an inhibition of Ca^{2+} mobilization are as follows. 1) Inhibition of IP_3 induce Ca^{2+} release. 2) Stimulation of sarcoplasmic and SR Ca^{2+}-ATPase. 3) Stimulation of Na^{+}-Ca^{2+} exchanger. Moreover, PA may act on other intracellular sites which lead to a decrease in Ca^{2+} sensitivity. The mechanism

may involve the stimulation or inhibition of several enzymes such as 1) inhibition of PDE, 2) inhibition of PKC enzyme, 3) stimulation of several protein kinases; and 4) stimulation of MLC phosphatase. However, the precise mechanism for the relaxing effect of PA is awaited to be determined.