The mechanisms and actions of parthenolide on platelet 5-HT secretion *in vitro*: indicating a possible pathogenesis of the migraine

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Abstract

Extracts of the medicinal herb feverfew and its active compound, parthenolide, have been determined to have anti-inflammatory, anti-migraine, and anti-cancerous effects, though the biochemical pathways involved have not been fully characterized. Both inhibit human platelet aggregation and serotonin (5-HT) secretion and are hypothesized to inhibit the protein kinase C (PKC) pathway. PKC mediates the phosphorylation of the 5-HT transporter in platelets and induces 5-HT secretion when activated. A platelet-based model was used to mimic the behavior of 5-HT neurons, and PKC activation was measured by the amount of 5-HT secreted. 5-HT secretion was measured using a comparison between 5-HT concentrations in platelet-rich plasma (PRP) and platelet-poor plasma (PPP): if PKC was inactivated and the treated platelets retained 5-HT, then PRP had a greater amount of 5-HT then PPP, if PKC was activated and 5-HT was secreted, then PRP and PPP had similar 5-HT concentrations. This study, using this model, indicates that platelets retain 5-HT when unclotted and release 5-HT when aggregated, thereby verifying the model appropriate for measuring platelet 5-HT secretion. This model also indicates that phorbol myristate acetate (PMA) induces platelet 5-HT secretion. Because aggregation of platelets and treatment with PMA have been correlated very strongly to PKC activation, this model is shown to be an accurate in-vitro model of PKC activation. This model suggests that parthenolide inhibits the PKC pathway, preventing 5-HT secretion; therefore the PKC pathway may be overactive in inflammatory diseases, certain cancers, and migraines, and drugs treating these diseases should target the PKC pathway.

Introduction

Feverfew (*Tanacetum parthenium*), an originally Balkan member of the Compositate family, has been used for millennia: first used by the ancient Greeks and early Europeans as an antipyretic, a repellant of insects, and to treat bites and stings. It now grows in North and South America, Europe, North Africa, China, Japan, and Australia. The first report of *T. parthenium* leaves as a treatment of migraines was made in 1978. (Kemper 1999) *T. parthenium* has been tested in 6 published, randomized, multicenter, placebo-controlled, double-blind studies and was shown to significantly decrease the severity and frequency of migraines in five of them (Johnson et al., 1985; Murphy et al., 1988; De Weerdt et. al., 1996; Palevitch et. al., 1998; Pfaffenrath et al., 2002; Diener et al., 2005). Two meta-analysis have been performed on these data: one found that feverfew significantly prevents the migraine, the other found that feverfew did not significantly prevent the migraine. Both are missing the 170 subjects from Diener 2005 (Ernst and Pittler, 2000; Pittler and Ernst, 2004). *T. parthenium* is also hypothesized to treat cancer and act as an anti-inflammatory; therefore it is used to treat rheumatoid arthritis and other inflammatory diseases (Pattrick et. al., 1989; Berry 1984; Johnson 1984; Wu et al., 2006; Parada-Turska et. al., 2007; Zunino et. al., 2007; Guzman and Jordan, 2005; Wen et. al., 2002). The mechanisms underlying these effects are unknown. Potentially active chemicals in *T. parthenium* include the sesquiterpene lactones: parthenolides, canin, artecanin, and santamarin; the flavonoid glycosides: luteolin, tanetin, apigenin, and 6-hydroxy-flavanols; the sesquiterpenes and monoterpenes: camphor, borneol, germacrene, and pinenes; polyacetylenes; pyrethrin; melatonin; and tannins (Kemper 1999). One of the identified active chemicals in *T. parthenium* is parthenolide, a sesquiterpene lactone composing 1-3% of *T. parthenium*, varying with geographic

origin. Parthenolide is the most abundant sesquiterpene lactone in *T. parthenium* and is thought to be its most active chemical constituent (Kemper 1999). The anti-inflammatory, anti-tumor, and anti-migraine properties of parthenolide have been studied, although not extensively (Groenewegen and Heptinstall, 1990; Wu et al., 2006). Both parthenolide and *T. parthenium* have been found to inhibit platelet release of pro-inflammatory chemicals, including serotonin (5-HT) and arachidonic acid (Heptinstall et. al. 1985; Groenewegen and Heptinstall, 1990). Both agents produce results with marked similarities between them, indicating that parthenolide may be the active compound in *T. parthenium* pertaining to platelet secretion of 5-HT (Groenewegen and Heptinstall, 1990; Kemper 1999; Piela-Smith and Liu, 2001; Sweeney, 2005).

Platelets store 5-HT in dense granules, which are secretory organelles containing 5-HT, adenine nucleotides, ionized calcium, histamine, and epinephrine (Furie et. al., 2001). When platelets are activated, in normal hemostasis and thrombosis, dense granules are stimulated to release these inflammatory chemicals through a complex series of signal transduction pathways (Figure 1) (Flaumenhaft, 2003; Rao and Gabbeta, 1999). The first two known pathways, the protein kinase C (PKC) and the Ca^{2+} pathways, are stimulated by epinephrine, ADP, thrombin, platelet activating factor, thromboxane, and collagen, which in turn induce the phosphorylation of PIP_2 (phosphatidylinositol 4,5-bisphosphate). PIP_2 is then hydrolyzed by various isoforms of PLC (phospholipase C) into DAG (diacylglycerol) and IP_3 (inositol trisphosphate). DAG activates the PKC signal transduction pathway, which phosphorylizes pleckstrin (P47) (Abrams 1995). Pleckstrin is a protein highly correlated to the secretion of 5-HT from dense granules in platelets (Polanowska-Grabowska, 1999). IP_3 stimulates the secretion of Ca^{2+} which leads to another independent signal transduction pathway activating dense granules (See Figure 1) (Flaumenhaft, 2003; Rao and Gabbeta, 1999). Both the Ca^{2+} and the PKC pathways result in the secretion of 5-HT from platelet granules, yet they are independent of each other (Turetta et. al., 2002; Coorssen et. al., 1990; Anderson and Horne, 1992; Rozenvayn and Flaumenhaft, 2002). Additional pathways include soluble NEM-sensitive attachment protein receptors (SNARE) proteins and the cytoskeleton (Flaumenhaft, 2003). The final pathway relevant to parthenolide and feverfew is the NF-kB (nuclear factor kappa-B) protein. Protein kinase D, a subset of PKC, phosphorylizes IKKβ (Storz and Toker, 2003). The IKK complex deactivates IkB, which is an inhibitor of NF-kB. NF-kB regulates the expression of genes involved in inflammation, autoimmune response, cell proliferation and apoptosis when stimulated by exposure to inflammatory cytokines, UV light, reactive oxygen species, or bacterial and viral toxins (Simon-Aldrich). With the inactivation of IkB, NF-kB enters the nucleus and acts as a promoter for the transcription and translation of pro-inflammatory compounds, including 5-HT (Kwok et. al., 2001). Because platelets share much of their morphology, biochemistry, and physiology with serotonergic neurons and because the blood brain barrier impedes research on the brain, platelets may be used as models of serotonergic neurons concerning 5-HT (D'Andrea et al. 2004; Lesch, 1993). Parthenolide has been hypothesized to affect platelet or neuronal secretion of 5-HT *in vivo*, therefore experiments involving parthenolide are well represented *in vitro* using platelets.

Groenewegen and Heptinstall (1990) found that extracts of the *T. parthenium* inhibit human platelet aggregation and secretion of 5-HT induced by several platelet aggregators: phorbol ester PMA (phorbol myristate acetate), ADP, arachidonic acid, collagen, the thromboxane mimetic U46619, the calcium ionophore A23187, the diacylglycerol analogue OAG, and epinephrine. They also found that parthenolide and *T. parthenium* extract were most effective as inhibitors of PMA-induced platelet aggregation. PMA activates the protein kinase C pathway in platelets and neurons and aggregates platelets (Coorssen, 1990). Because Groenewegen and Heptinstall found that parthenolide inhibited PMA-induced platelet aggregation, the effects of parthenolide on 5-HT secretion may result from an inhibition of the protein kinase C pathway (Chapter 3, Carneiro et al 2006, Newton 1995). In support of this

hypothesis, parthenolide has been found to sensitize UVB-induced apoptosis in epidermal cells through the protein kinase Cζ and Cδ pathways (Won et al. 2005). Protein kinase C has also been hypothesized to be overactive in the spreading depression correlated with the migraine (Osten, 1996).

Parthenolide has been found to indirectly inhibit the pro-inflammatory pathway (NF-kB) (Pozarowski, et. al., 2003; Hehner, et. al., 1999). Kwok et al. (2001) found that parthenolide directly binds to and inhibits IKK which supports the hypothesis that parthenolide inactivates nuclear factor-kB (NF-kB). I propose that parthenolide also inhibits the protein kinase C in platelets, inhibiting the phosphorylation of pleckstrin leading to the secretion of 5-HT from platelet dense-granules (See Figure 1). If parthenolide inhibits 5-HT secretion from dense platelet granules through the PKC pathway, then parthenolide should inhibit the effects of PMA, a PKC activator. This study should indicate a potential pathway for the affects of parthenolide as an anti-inflammatory agent, a migraine preventive, and an anti-tumor agent, leading to a greater understanding of migraine pathogenesis and more efficient and precise treatments.

Materials and Methods

Blood collection

About 1.5 mL trunk blood, obtained from male Wistar rats (*Rattus norvegicus*) euthanized with an overdose of sodium pentobarbital for use in a study under the IACUC approved protocol "Functional consequences of increased TPH2 in mRNA expression in depression," was collected into 2mL eppendorf tubes containing 150μL acid citrate dextrose (sigma C3821-50ML) (Sánchez et al. 2003). The euthanization and decapitation of the rats and collection of the blood were performed by a qualified scientist. Each rat yielded from three to five uncoagulated 1.5mL blood samples. The blood at this stage may be stored for three days at 4° C before significantly differing from *in vivo* blood (Högman et al. 1999; Gibbons et al. 1982; Kahn et al. 1981).

Blood treatment

Blood from each eppendorf tube was aliquoted into multiple 250μL replicate samples (3-6) and each aliquot of whole blood was treated with parthenolide and/or PMA, or DMSO vehicle diluted in saline. To test for the effects of PMA on platelet release of serotonin, 25 μL PMA (0.2, 2, 10, or 20 μM) or DMSO vehicle diluted in saline (1:1000), was added to 250 μL of unclotted whole blood, samples were briefly vortexed and platelet-rich and platelet-poor plasma were then obtained as described below. The effect of parthenolide on platelet release of serotonin was examined by treating 250μL of unclotted whole blood samples with 25μL parthenolide (0.5, 5, or 50μM) or DMSO vehicle diluted in saline (1:500), briefly vortexing the samples, then obtaining platelet-rich and platelet-poor plasma. The effect of parthenolide with PMA on platelet release of 5-HT was tested by first treating 250μL of unclotted whole blood samples with 25μL parthenolide (0.5, 5, 50, 100μM) or DMSO vehicle diluted with saline (1:500), briefly vortexing the samples and allowing them to incubate for one minute, then treating the samples with 25μL PMA (10, 20μM) or DMSO vehicle diluted with saline (1:1000). The samples were then vortexed and platelet-rich and platelet-poor plasma were obtained as described.

Platelet-rich plasma and platelet-poor plasma

Following treatment of whole blood with PMA and/or parthenolide, whole blood samples were centrifuged at 22° C for 15 min at 1500 rpm (rotations per minute) to obtain platelet-rich plasma (PRP). Two 50μL aliquots were removed and stored at -80° C. The remaining PRP was centrifuged at 22° C for 30 min at 4000 rpm to obtain platelet-poor plasma (PPP), and another two 50μL aliquots were removed and stored at -80° C.

HPLC (high pressure liquid chromatography) analysis

The frozen 50μL samples were thawed at 22°C, and treated with 50μL of 20% perchloric acid in saline to obtain a 1:1 dilution of PRP or PPP with perchloric acid. The addition of perchloric acid denatures and precipitates the platelets, releasing their 5-HT into solution. The plasma was vortexed for one second, then centrifuged at 13,000 rpm for 4 minutes at 22°C to cause the denatured proteins to separate from the solution, leaving a supernatant on the surface. HPLC analysis was used to determine the concentrations of 5-HT and 5-HIAA (5-Hydroxy-3-indoleacetic acid) in each sample (Hirowatari et al. 2004; Pussard et al. 1996). The 5-HIAA concentration in the samples was measured to ensure that the measured 5-HT concentration did not include 5-HIAA, serotonin's catabolic product. 70μL of the supernatant of each sample was placed into an ESA 542 autosampler maintained at 4°C. 10μL of the supernatant from each sample was then injected into the chromatographic system. Chromatographic separation was accomplished using an integrated precolumn/column system containing of a guard cartridge attached to an Ultrasphere XL-ODS cartridge. The mobile phase consisted of 9.53 g/L KH_2PO_4, 20mg/L ethylenediaminetetraacetic acid in 13% methanol and electrochemical detection was accomplished using an ESA Coulochem II cell with electrodes set at -0.10 and + 0.55 V. The mean peak heights (pg/cm) of known concentrations of 5-HT and 5-HIAA standards were determined from the peak heights of two chromatographs run before and after each set of samples. Concentrations of 5-HT and 5-HIAA in the samples were determined based on peak heights measured using a computerized analysis system (EZ Chrom Elite for Windows, version 2.8), and these concentration of 5-HT and 5-HIAA in the plasma was expressed as pg/μL.

Statistics and analysis

The treatment effects of PMA and parthenolide on PRP when compared to PPP were determined by a single multifactor analysis of variance (ANOVA) test, which included all replications. When a treatment effect was found ($p<0.05$), a post-hoc Tukey HD test was performed to determine which trials drove the treatment effect. Using original data, the averages of each treatment separated into PRP and PPP were obtained and graphed, with error bars indicating the standard error of the mean. Platelet release of 5-HT into the plasma was reflected in a ratio between the amount of 5-HT found in the platelet-poor plasma and the platelet-rich plasma. When platelets retained 5-HT, PRP was significantly larger than PPP and when platelets released 5-HT, PRP and PPP were essentially the same.

Safety

Rat blood is classified as BSL 2 because it is a potential carrier and transmitter of zoonotic diseases and because it may elicit an allergic reaction; therefore, safety precautions are required. Treatments for the zoonotic diseases were available if the preventive measures outlined below failed. The lab was constantly well ventilated, and I was constantly wearing gloves, a laboratory coat, and goggles, as recommended by the

Animal Care Centre. The blood was stored in a biological refrigerator at -80°C when not being used. In the case of a spill or bodily contact, the blood was disposed of in a Biohazardous waste disposal and the area in contact with the blood was washed well with soap and water. A Qualified Scientist constantly supervised the experiment. The blood products and all equipment in contact with blood were disposed of in a Biohazardous Waste disposal, which was later autoclaved.

Results

Unclotted whole blood, which contains at least 50μL of an anticoagulant (EDTA or CDA) for each mL of blood, when separated into platelet-rich and platelet-poor plasma contains a significantly larger concentration of 5-HT in PRP than in PPP (Fig. 2). A single-factor analysis of variance (ANOVA), with an alpha equal to 0.05, found that the difference that exists between PRP and PPP, obtained from unclotted blood, has a 6.59×10^{-10} % chance of occurring by chance alone ($p<0.001$, $F_{crit}=4.043$, $F=81.39$). However, in clotted whole blood there was no statistical difference in the concentration of 5-HT in PRP than in PPP. An ANOVA, with an alpha equal to 0.05, found that the difference that exists between PRP and PPP, obtained from clotted blood, has a 60.3% chance of occurring by chance alone ($p=0.603$, $F_{crit}=4.047$, $F=0.275$), therefore the average values are statistically the same. Three rats were euthanized to obtain the data for the unclotted averages and two rats were euthanized to obtain the data for the clotted averages.

In whole blood treated with PMA, PMA had no effect on plasma 5-HT concentrations in PRP at any concentration tested (0.2, 2, 20μM), while PPP demonstrated a significant concentration-dependent change in 5-HT concentration with the different treatments of PMA (Fig. 3). All PRP samples obtained from whole blood treated with PMA (0, 0.2, 2, 20μM) were compared using an ANOVA, and the PMA treatments were found to have no significant effect. The chance that the differences between the averages of PRP occurred only by chance is 81% ($p=0.81$, $F_{crit}=6.59$, $F=0.323$). PMA therefore has no effect on the concentration of 5-HT in PRP. All PPP samples obtained from whole blood treated with PMA (0, 0.2, 2, 20μM) were compared using an ANOVA, and the PMA treatments were found to have a significant effect. The chance that the differences between the averages of PPP occurred only by chance is 1.87×10^{-7} % ($p<0.001$, $F_{crit}=3.587$, $F=167.05$). A Tukey HSD test was performed as a post-hoc statistical test, and the treatments of 20μM and 2μM were found to drive the ANOVA. All samples for these ANOVA's were obtained from one rat. A verification of these results was performed with the addition of 25μL of DMSO vehicle diluted in saline (1:500) (Fig. 5). The ANOVA between PRP had a $p=0.249$ and the ANOVA between PPP had a $p=0.3964$; therefore PMA was not found to have a treatment effect by increasing 5-HT concentration in PPP. Yet the standard errors of the mean for this study do not intersect for the PPP, suggesting that PMA continued to increase 5-HT concentration in PPP with the addition of 25μL of DMSO vehicle diluted in saline.

Parthenolide by itself (0, 5, 50μM) was found to have no significant effect on the concentration of 5-HT in both PRP and PPP (Fig. 4). An ANOVA determining the effect of parthenolide on 5-HT concentration in PRP, obtained from whole blood treated with parthenolide (0, 5, 50μM), found that the chance of the differences between the treatments occurring purely by chance is 72.4% ($p=0.724$, $F_{crit}=3.982$, $F=0.333$). Therefore, parthenolide has no treatment effect on 5-HT concentration in PRP. The ANOVA performed between the platelet-poor plasma's found that the chance of the differences between the treatments occurring purely by chance is

9.98% ($p=0.100$, $F_{crit}=3.739$, $F=2.73$). Therefore, parthenolide has no treatment effect of 5-HT concentration in PPP either. The whole blood samples were obtained from three rats.

Parthenolide was also found, by an ANOVA, to have no significant effect of the concentration of 5-HT in both PRP and PPP when the whole blood was treated with 10μM or 20μM PMA (Fig. 6 and Fig. 7). Yet, the standard error of the mean for 5μM, 50μM, and 100μM do not intersect with the standard error of the mean for 0μM or 0.5μM, suggesting that parthenolide might have an effect on the concentration of 5-HT in PPP for 10μM PMA treatments (Fig. 7). The ANOVA performed on PRP, obtained from whole blood treated with 25μL of 20μM PMA in addition to parthenolide (0, 0.5, 5, 50, 100μM), had a $p=0.679$, indicating that the differences between the averages of PRP are extremely likely to have occurred by chance alone and that parthenolide has no treatment effect on 5-HT concentration in PRP. The ANOVA performed on PPP, obtained from whole blood treated with 25μL of 20μM PMA in addition to parthenolide (0, 0.5, 5, 50, 100μM), had a $p=0.080$, indicating that parthenolide had a non-significant increase on the concentration of 5-HT in PPP, which might become significant with an increase in sample size, but that parthenolide has no statistically significant treatment effect on PPP. Three rats were euthanized to provide the whole blood samples for these treatments. The ANOVA, used to test the effects of parthenolide and PMA on 5-HT concentration in PRP, performed on PRP, obtained from whole blood treated with 25μL of 10μM PMA in addition to parthenolide (0, 0.5, 5, 50, 100μM), had a $p=0.357$, indicating that the differences between the averages are likely to have occurred by chance alone and that parthenolide has no treatment effect on 5-HT concentration in PRP. The ANOVA performed on PPP, obtained from whole blood treated with 25μL of 10μM PMA in addition to parthenolide (0, 0.5, 5, 50, 100μM), had a $p=0.352$, indicating that the differences between the averages are likely to have occurred by chance alone and that parthenolide has no statistically significant treatment effect of 5-HT concentration in PPP. An ANOVA performed on PPP, obtained from whole blood treated with 25μL of 10μM PMA in addition to parthenolide (0, 0.5, 5, 50, 100μM), comparing a control (0μM PMA, 0μM parthenolide) and one sample (10μM PMA, 0μM parthenolide) had a $p=0.300$, indicating that PMA, without parthenolide, had no significant treatment effect on 5-HT concentration in PPP. The standard errors of the mean for these two treatments suggest, however, that the trend indicated in previous studies continued in this study. Two rats were euthanized to provide the whole blood samples for these treatments. The standard errors of the mean between treatments 1 and 2 and 3, 4, and 5 in figure 7 do not intersect, providing evidence for pathenolide's ability to negate PMA's effects on PPP. Although these data are not significant, they suggest a trend supporting previous experiments (Groenewegen and Heptinstall, 1990) and my hypothesis.

Discussion and Conclusions

The above data characterize a platelet-based model for measuring platelet release of 5-HT and the activation of platelet and neuronal molecular pathways leading to the granular secretion of inflammatory hormones, including 5-HT. These data indicate that clotting blood causes platelet release of 5-HT and that unclotted blood retains 5-HT, supporting knowledge of platelet behavior *in vivo* (Furie et. al. 2001, Flaumenhaft, 2003; Rao and Gabbeta, 1999). These data also indicate that PMA induces platelet release of 5-HT, which supports findings by Groenewegen and Heptinstall (1990) and Coorssen (1990). Both of these conclusions verify that this platelet-based model accurately represents platelets *in vivo*; therefore these data characterize a model for measuring platelet release of 5-HT. Given that this model accurately represents platelet behavior in whole blood, these data indicate that parthenolide has no effect on normal platelet behavior

when platelet secretion pathways are inactive. Finally, these data indicate, though not significantly, that parthenolide inactivates the effects of PMA when parthenolide is in high concentrations and PMA is in low concentrations (10μM). This trend suggests that parthenolide inhibits the PKC pathway and warrants further study.

Under normal conditions, platelets store 5-HT in dense granules and release 5-HT when stimulated by clotting factors. In the presence of anticoagulants, 5-HT is retained within the (Furie et. al., 2001; Flaumenhaft, 2003; Rao and Gabbeta, 1999). The process of coagulation in whole blood and *in vivo* stimulates the release of platelet-activating hormones (including epinephrine, ADP, thrombin, PAF, thromboxane, and collagen), which activate inflammatory hormone secretion from platelets (Fig 1). Coagulation and platelet aggregation therefore stimulate the secretion of 5-HT. In the present study, 5-HT concentration in unclotted blood samples,was found to be significantly larger in platelet-rich plasma than in platelet-poor plasma. According to the predicted model, these data indicate that 5-HT was retained in platelets and was spun out of solution in the platelet-poor plasma. In line with the prediction that coagulation induces platelet release of serotonin, in this study, clotted blood samples yielded no difference in 5-HT concentration between PRP and PPP. According to the model, these data indicate that 5-HT was secreted from platelets due to platelet aggregation and blood coagulation (the only difference between the trials was the addition of an anticoagulant). These data and their implications provided by the platelet-based model are consistent with the studies above, indicating that this model is an accurate representation of platelets *in vivo* and that this model may be used in further experiments.

PMA, in Groenewegen and Heptinstall (1990) and in Coorssen (1990), has been found to induce the secretion of 5-HT from platelets and to induce coagulation of blood. Coorssen (1990) also determined that PMA activates the PKC pathway in platelets and neurons. In this study, PMA did not induce coagulation in whole blood samples, possibly due to the anticoagulant (CDA). CDA was used in this study to ensure that the whole blood and the platelets mimicked whole blood and platelets in vivo, thereby increasing the relevancy and the accuracy of the model. CDA also ensured that 5-HT secretion was only a result of the treatment, not of aggregation. Because CDA does not interfere with the PKC pathway, CDA does not influence PMA's activation of the PKC pathway and its ability to induce the secretion of 5-HT; therefore, CDA was used as an anticoagulant. In this study, PMA was shown to significantly increase the concentration of 5-HT in PPP while having no effect on the concentration of 5-HT in PRP. Assuming that the model explained in the methods and introduction is accurate, these data indicate that PMA induces platelet secretion of 5-HT. Again, these data and their implications provided by the platelet-based model, explained in the introduction and methods, are consistent with the studies described above, further indicating that this model is an accurate representation of platelets *in vivo*. Given that all results and conclusions previously tested using other models were replicated using this platelet-based model, this study has characterized a model for measuring platelet-release of 5-HT and for measuring platelet activation of inflammatory secretion pathways in platelets. This model may be used as a representation of platelets in *R. norvegicus* and human whole blood in further studies.

In this study, parthenolide was found to have no significant effect on the concentration of 5-HT in PRP or PPP. Both platelet-rich and platelet-poor plasma retained their 5-HT, as expected with unclotted blood, without being altered by the treatment of parthenolide. These data indicate that the platelets retained their 5-HT and that no inflammatory secretion pathways were activated within the platelet. These results and conclusions support parthenolide's predicted qualities: that it would inhibit inflammatory pathways, not activate them (Groenewegen and Heptinstall, 1990; Kemper 1999).

Parthenolide was found to have no statistical significance on the effects of PMA on 5-HT concentration in PRP and PPP. Blood without PMA and parthenolide, simply containing controls of DMSO diluted in saline (25μL for both controls, 1:1000 and 1:500) behaved as normal, unclotted blood, although the differences between PRP and PPP were not statistically significant, indicating that more replicates are required in further investigation. PMA treatment of whole blood containing a control of 0μM parthenolide also resulted in similar 5-HT concentrations in PRP and PPP as PMA treatment of whole blood (Fig 3, 6 and 7); however the differences between treatments in PPP were not statistically significant and a large variety existed between individual samples, indicating that more replicates are required in further investigation. Parthenolide (0.5-50μM) had no effect on 20μM PMA induced platelet release of 5-HT, possibly due to a very strong activation of PKC by PMA which could not be reversed by parthenolide. In order to resolve this potential problem, further tests were conducted with a lower dose of PMA (10 uM). These tests yielded data suggesting that parthenolide may inhibit the effect of PMA on 5-HT secretion, although the effects of parthenolide were not statistically significant. Because neither of the controls in this experiment were statistically the same as previous controls, these data may not be accepted as indications of the effect of parthenolide on platelets *in vivo*. Further testing is necessary to clarify these tests. However, the trends of this study match hypothesized results and suggest that parthenolide inhibits 5-HT secretion from platelets through the PKC pathway; therefore, parthenolide may inactivate the PKC pathway, decreasing spreading depression and the inflammation of the meninges and blood vessels, and thus decreasing the frequency and severity of the migraine. PKC may be further hypothesized to be overactive in migraine, inflammatory diseases, and possibly in certain forms of cancer. These data and possible conclusions warrant further study.

Possible sources of error in this study and flaws in these methods include the fact that this study assumes that PMA activates only the PKC pathway in platelets without affecting PKD or PIP_2, which would activate alternate pathways, including Ca^{2+} or NF-κB. All evidence found indicates that these two assumptions are correct, yet the actions of PMA on platelet secretion of 5-HT have not yet been fully characterized and PMA may be found to influence PKD or PIP_2 (Coorssen, 1990; Storz and Toker, 2003). PMA treatment of whole blood led to a significantly larger 5-HT concentration in platelet-poor plasma than in platelet-rich plasma (Fig. 3,5, and 6). These data are unexplained by the model used in this study; 5-HT was assumed to be contained only in platelets, not in the whole blood, PPP cannot contain more 5-HT than PRP. Whole blood was treated, then spun into PRP, then spun into PPP, so all components of whole blood were present in the eppendorf tube and PMA is assumed to have acted on some of these components during the spin of PRP into PPP, inducing 5-HT release and an increase in 5-HT concentration in PPP (30 min). In order to remove this source of error, the whole blood-CDA solution should be spun into PRP (15 min, 1500 rpm, 37°C) before treatment. PRP should then be aliquoted into 250μL samples and be treated as described in the methods section under *Blood treatment*. Preliminary experiments using this method of treating PRP versus whole blood to determine the effect of PMA on 5-HT concentration in PRP and PPP have been performed and the treatment of PMA has been found to have a significant effect on the concentration of 5-HT in PPP (Fig 8). The ANOVA test for PRP comparing four treatments of PMA (0, 0.2, 2, 20μM) yielded a $p=0.809$, indicating that PMA has no effect on 5-HT concentration in PRP. The ANOVA test for PPP yielded a $p=0.036$, indicating that PMA has a significant effect on 5-HT concentration in PPP and that PMA induces platelet secretion of 5-HT. A Tukey HSD post-hoc test found that the 20μM PMA treatment drove the significance of the ANOVA test. These methods should be repeated to determine the effect of parthenolide on 5-HT concentration and secretion and to determine the effect of feverfew on PMA induced 5-HT secretion. This preliminary study indicates that the

trend suggested by previous studies, that parthenolide inhibits 5-HT secretion induced by PMA, will be continued through further experimentation.

Future experiments are suggested, using the platelet-based model described and used in the study described above, to isolate the PMA activated pathway which may be inactivated by parthenolide and confirm that parthenolide inhibits the PKC pathway, not an alternate pathway leading to platelet 5-HT secretion. Replicates in which PRP is treated should also be performed for studies in which whole blood was treated. Using this revised model based on PRP rather than on whole blood, further experiments should be implemented in the same pattern as was used for PMA with a different platelet activator, Ca^{2+}. Ca^{2+} is appropriate to cross test parthenolide because it induces platelet release of serotonin via an alternate pathway than the PKC pathway. If parthenolide only inactivates the PKC pathway, then Ca^{2+} induced platelet 5-HT secretion should be uninhibited with or without the addition of parthenolide (Fig. 1). The addition of these studies and the alteration of the methods as described above should strengthen the conclusions of this study. This study characterized a model for measuring platelet secretion of 5-HT and the activation of inflammatory secretion pathways in platelets, while suggesting that parthenolide inhibits the PKC pathway. Parthenolide's possible ability to inactivate the PKC pathway indicates that the PKC is overactive in patients with migraine and that parthenolide decreases spreading depression and the inflammation of cerebral blood vessels and thus the frequency and severity of the migraine. PKC may be further hypothesized to be overactive in migraine, inflammatory diseases, and possibly in certain forms of cancer.

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Appendix

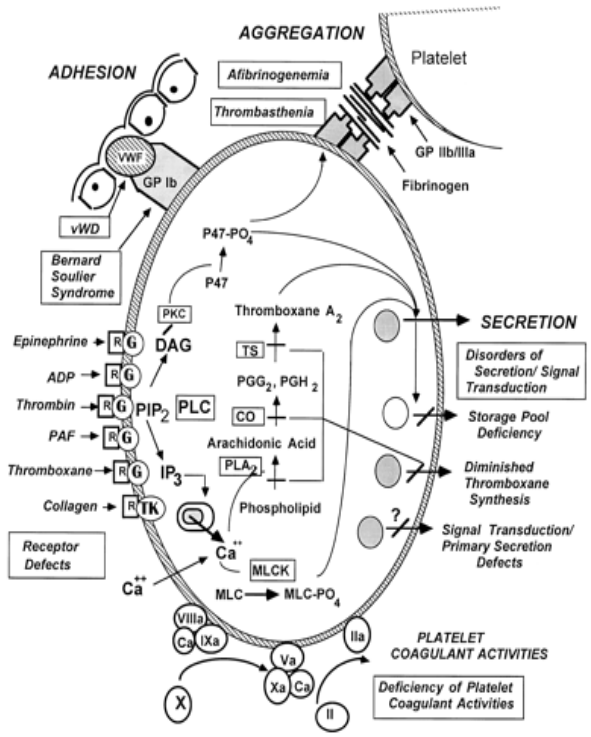

Figure 1. Obtained from Rao and Gabbet 2000. "Schematic representation of normal platelet responses and the congenital disorders of platelet function. Some of the platelet-mediated coagulation protein interactions are also shown. Coagulation proteins are shown by Roman numerals, with the activated forms designed by the letter a. The arrows designate conversions of zymogens to enzymes. CO indicates cyclooxygenase; DAG, diacylglycerol; G, GTP-binding protein; IP_3, inositol trisphosphate; MLC, myosin light chain; MLCK, myosin light-chain kinase; PIP_2, phosphatidylinositol bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLA_2, phospholipase A_2; R, receptor; TK, tyrosine kinase; and TS, thromboxane synthase."

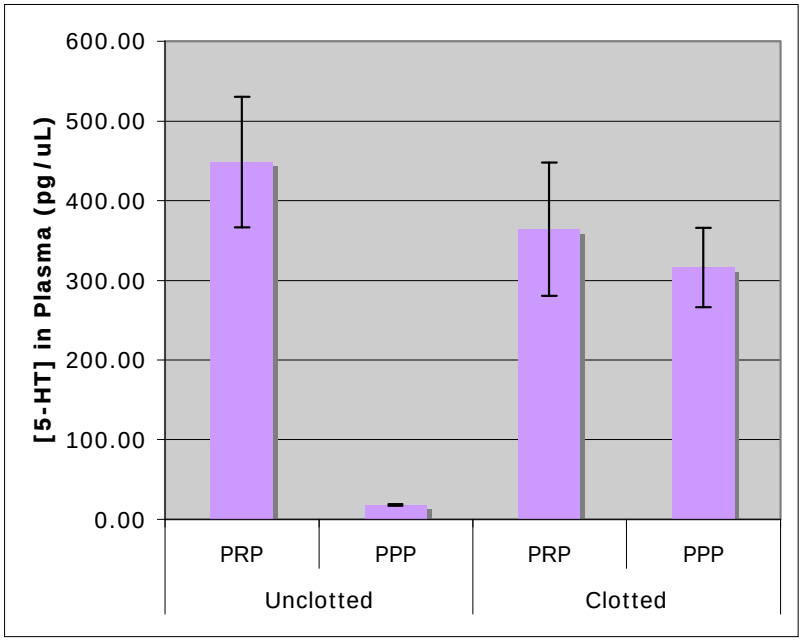

Figure 2. The effects of clotting on 5-HT secretion, shown as the difference in 5-HT concentration (pg/μL) between PRP and PPP in clotted and unclotted blood. The error bars indicate the standard error of the mean. PRP and PPP data from unclotted blood represent 50 samples obtained from 3 rats. PRP and PPP data from clotted blood represent 49 samples from 2 rats.

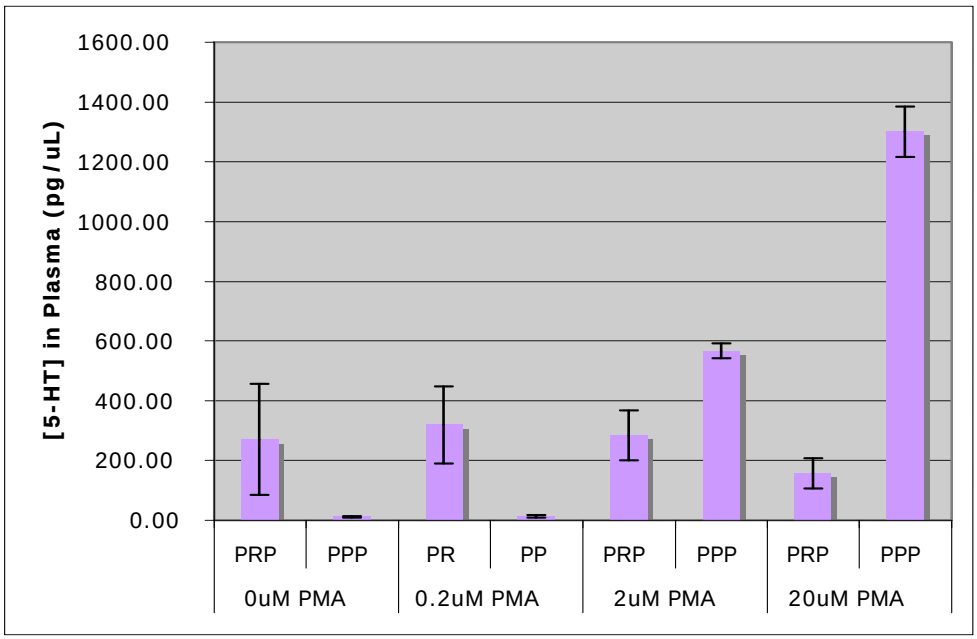

Figure 3. The effects of PMA on 5-HT secretion, shown as the difference in 5-HT concentration (pg/μL) between PRP and PPP. The error bars indicate the standard error of the mean. The PRP data represent 8 samples from one rat. The PPP data represent 15 samples from one rat.

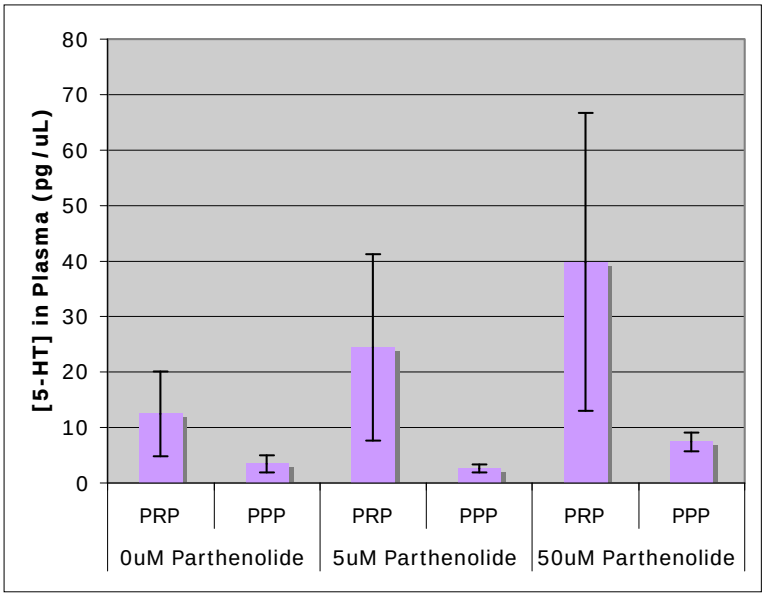

Figure 4. The effects of parthenolide on 5-HT secretion, shown as the difference in 5-HT concentration (pg/μL) between PRP and PPP. The error bars indicate the standard error of the mean. All treatments contained 25μL of a DMSO vehicle and concentration control diluted in saline (1:1000). PRP data represent 14 samples from 3 rats; PPP data represent 17 samples from 3 rats.

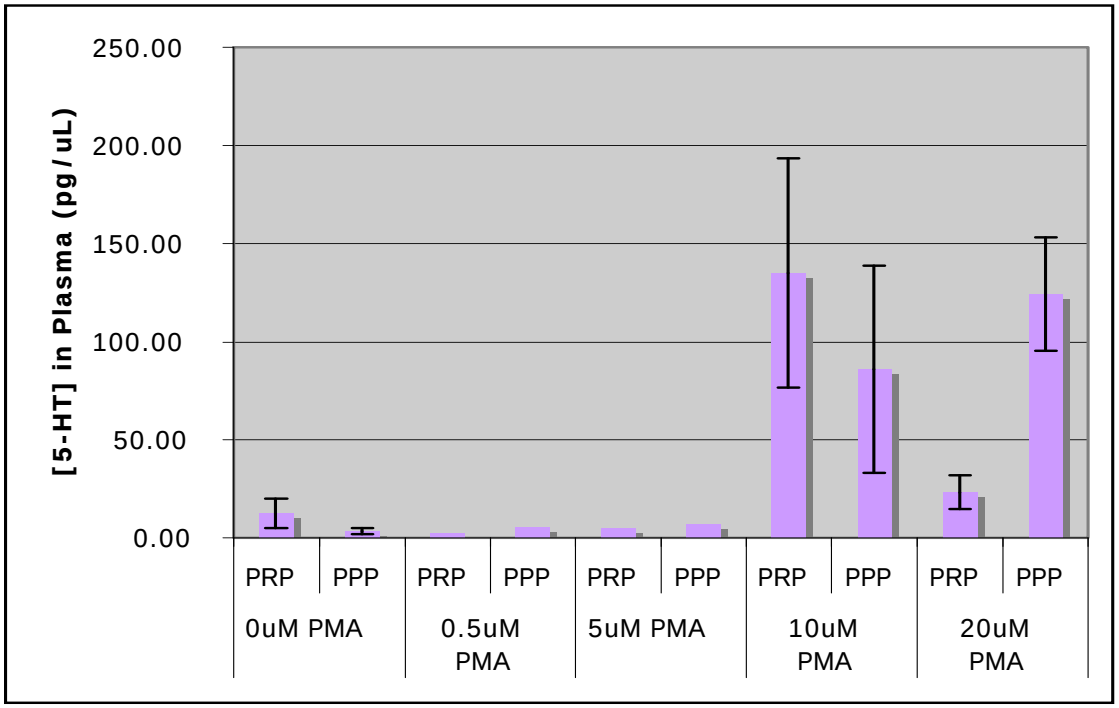

Figure 5. A verification of the effects of PMA on 5-HT secretion, shown as the difference in 5-HT concentration (pg/μL) between PRP and PPP. The error bars indicate the standard error of the mean. All treatments contained 25μL of a DMSO vehicle and concentration control diluted in saline (1:500). PRP data represent 25 samples from 4 rats; PPP data represent 29 samples from 4 rats.

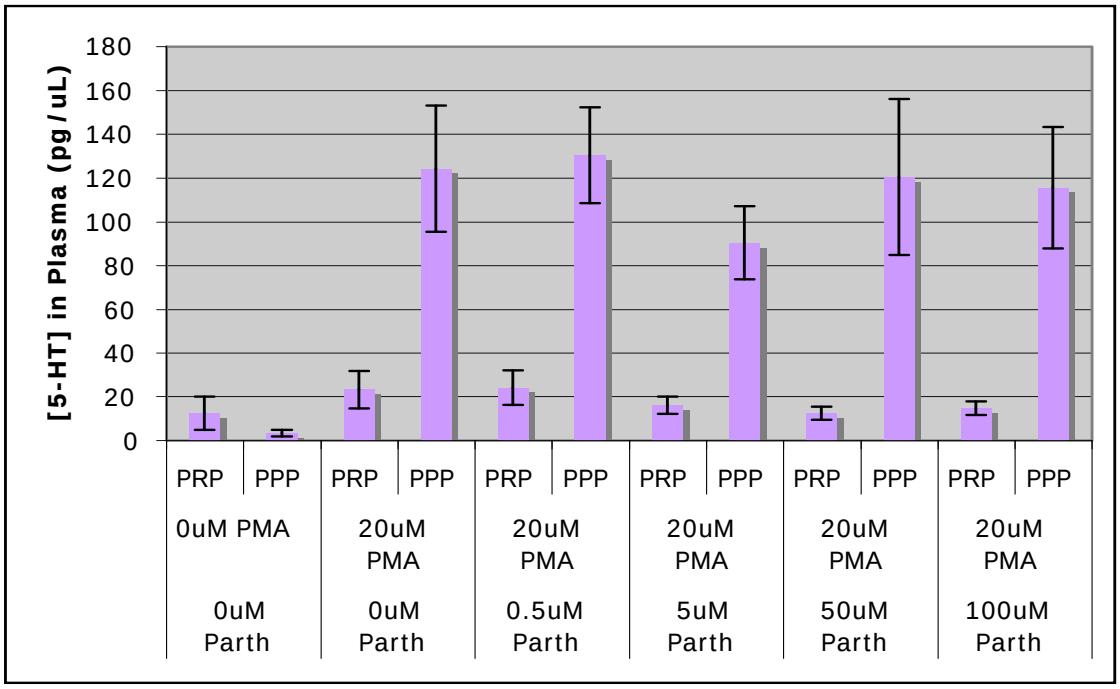

Figure 6. The effects of parthenolide on PMA induced 5-HT secretion, shown as the difference in 5-HT concentration (pg/μL) between PRP and PPP. The error bars indicate the standard error of the mean. Parth stands for parthenolide. PRP data represent 38 samples from 3 rats; PPP data represent 55 samples from 3 rats.

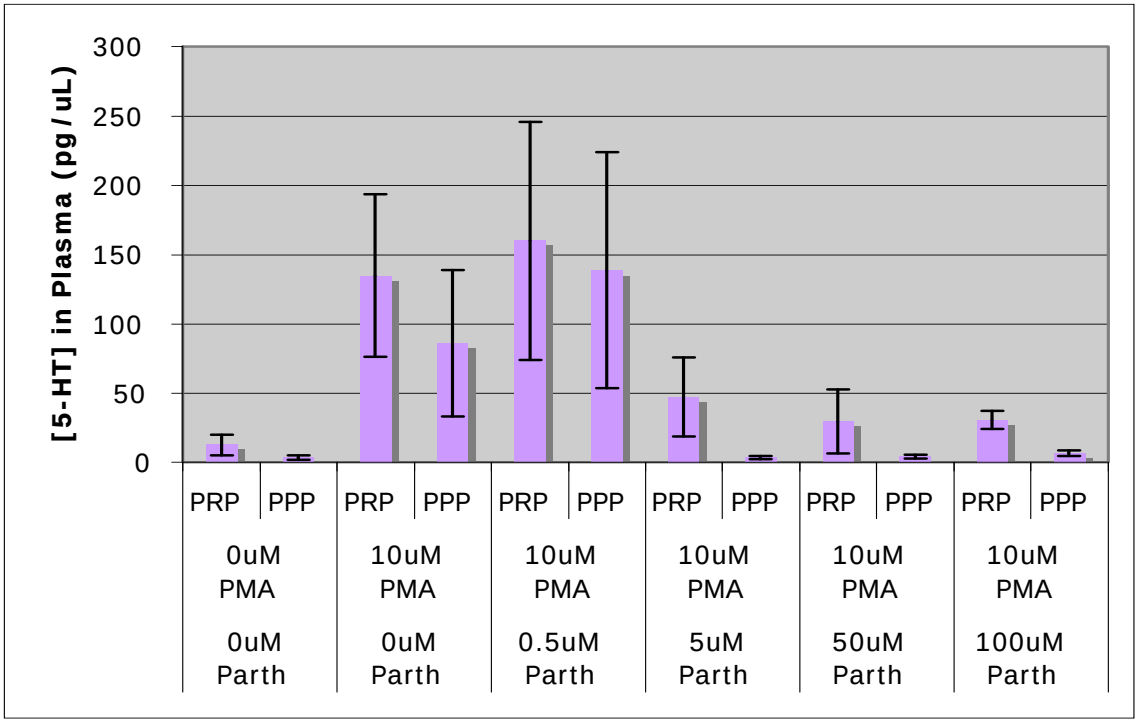

Figure 7. The effects of parthenolide on PMA induced 5-HT secretion, shown as the difference in 5-HT concentration (pg/μL) between PRP and PPP. The error bars indicate the standard error of the mean. Parth stands for parthenolide. PRP data represent 30 samples from 2 rats; PPP data represent 34 samples from 2 rats.

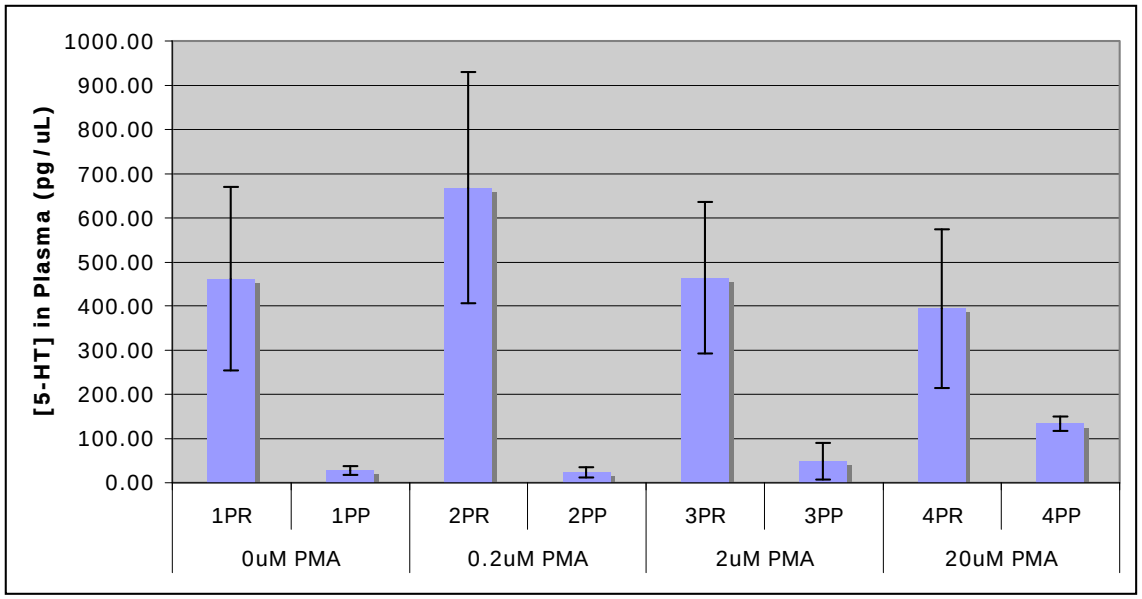

Figure 8. (Preliminary study) The effects of PMA on 5-HT secretion, shown as the difference in 5-HT concentration (pg/μL) between PRP and PPP. Treatments of 25μL PMA (in varying concentrations: 0μM, 0.2μM, 2μM, 20μM) were performed on platelet-rich plasma rather than on whole blood. The error bars indicate the standard error of the mean. PRP data represent 16 samples from one rat; PPP data represent 12 samples from one rat.