Receptor(s) activation and cAMP elevation by the PGE1 analogue, Misoprostol in cultured human fibroblast cell line (W138).

Sankar P. Mitra

Sepracor Inc, 84 Waterford Drive, Marlborough, MA 01752, USA

Key Words:

Misoprostol, Fibroblast, PGE1, W138, Colchicine, cAMP, G-proteins, EP- receptors, Pertussis Toxin, Cholera Toxin, Forskolin, Adenylate Cyclase, G-protein.

Address of correspondence:

Sankar P. Mitra Ph.D, 102, Vassar street, Worcester, MA 01602
Phone: 508-752-3554.
Email: Sankar_mitra@msn.com or smitra3554@gmail.com.

Abstract

Misoprostol is a racemate of synthetic PGE1 analog and an agonist to the EP receptors. The drug elevates cAMP level dose-dependently (EC_{50} ~ 7.0 μM) in human lung fibroblast cell line, W138. Both free acid and its methyl ester are seen to be equally potent in that act. The specific PGE1 receptor (EP2) antagonist AH6809 inhibits the effect of cAMP elevation (EC_{50} ~ 10μM) almost ~ 90 %. Pre-treating the cells with Pertussis toxin (PTx) enhances the cAMP level considerably whereas Cholera toxin (CTx) instead of elevating it lowers the level by ~ 25 % in presence of Miso. The events characteriscally indicate that Miso works by activating both Gs and G_i associated with its receptor(s). The anti-mitotic drug Colchicine lowers the basal production of cAMP but also suppresses the elevating effect by Miso. On the other hand the action of Forskolin (FSK) in its presence shows only an additive effect. All these facts strongly suggest that either the agonist interaction may proceed via a single receptor which is promiscuously associated with both stimulatory (G_s) and inhibitory (G_i) G -proteins or else the W138 cell line expresses both EP2 and EP3 (subtype EP3α) which are respectively connected to them while acting simultaneously.

Abbreviations: Misoprostol (Miso), Prostaglandin E (PGE), Pertussis toxin (PTx), Cholera toxin (CTx), Forskolin (FSK), Interleukin (IL), Tumor necrosis factor (TNF), Interferon (IFN), G-protein coupled receptor (GPCR), Inhibitory G-protein (G_i), Stimulatory G-protein (G_s), Adenylate Cyclase (AC).

Introductions

Misoprostol is a synthetic PGE1 analogue (Fig – 1); first developed for treating the peptic ulcer but later used for several other clinical purposes that includes induction of labor or early abortion of the fetuses[1]. In recent days, it finds far more beneficial applications for example, curing

of inflammatory diseases as well as for osteo-arthritis[2 - 4]. As reported by different workers, Miso inhibits the mitogenic actions of IL-1, TNF- α and β including the production of IFN – γ [5 - 9]. On the other hand it enables to enhance the IL - 6 generation [6]. Like PGE1, its actions are mediated through the binding of specific cell surface receptors expressed in different areas of the body, causing either the relaxation or contractions of smooth muscles. As per additional action, the drug also modulates neuronal actions by inhibiting the release of different neurotransmitters. It is already known that many prostanoids can sensitize the sensory neuronal fibers in response to various noxious stimuli thereby generating fevers, pain or any other discomforts. Additionally, these prostanoids are linked to different cellular and physiological functions like apoptosis, cell differentiation, oncogenesis or regulating the platelet functions in both ways [10 - 17]. The receptors of prostanoids belong to the GPCR super family with seven trans-membrane strands spanning the cell surface bilayer. Until now eight types of prostanoid receptors have been identified; among them only four belongs to the EP class (EP1, EP2, EP3 & EP4). Within the EP subtypes, only the EP3 offers four other extra subclasses EP3α, EP3β, EP3γ & EP3δ [18].The overall biologic responses of the EP receptors occur either by intracellular $[Ca^{+2}]_i\uparrow$ elevation through EP1 or by the modulation of cAMP level working via either EP2, EP3 or EP4 receptors expressed on the cell surface [19]. The previous reports indicate that Miso binds to the same receptor(s) of PGE1. Having structural similarity to PGE1, it acts as an agonist to the EP1, EP2 / EP4 and EP3 receptors (Fig – 1) [21, 22]. The cAMP generation by PGE1 is known due to the activation of G_s which occurs through its binding at the second loop of EP2 [21 - 23]. On the other hand, EP3α is anchored with G_i therefore its activation tends to lower the level of stimulated intracellular cAMP production [22, 25]. But the dual action of Miso is currently observed while interacting with the human fibroblast cell line, W138.

As per chemical structure, both PGE1 and Miso bears a strong resemblance except at the C15 and C16 positions (Fig – 1) [25]. Interestingly, changing the position of – OH from C15 of PGE1 to C16 within chemical structure of the drug reduces the side effects of diarrhea [26 - 28]. The addition of – CH_3 group at same C16 offers extra stability in comparison to its natural analog, PGE1 [26 - 31]. The commercially available synthetic Miso exists as a racemic mixture of four stereo-isomers of which only one, (Methyl 7- {(1*R*, 2*R*, 3*R*)-3-hydroxy-2-((*S, E*)-4-hydroxy-4-methyloct-1-enyl)-5-oxocyclopentyl} heptanoate) is known to be physiologically active and acts via the EP receptor(s) whereas the other three remain as neutral [29 - 32]. The drug is often claimed to be more physiologically active in free acid (- CO_2H) form than its methylated ester (- CO_2CH_3). It has a longer half-life ($t_{1/2}$) than its natural analogue, PGE1[1]. Usually, near nano-molar ($\sim 10^{-9}$ M/L) ranges, the drug offers beneficial roles like, protecting tissue damages or preventing any ulceration whereas at

higher concentrations (~ 100 x 10^{-6} M/L) around micro molar doses it loses the favorable effects therefore often acting adversely by worsening the ailments [1, 32 - 34]. When introduced at maximum therapeutic doses its level in plasma reaches at best to the concentration of 0.7 ng / ml (~ 3.0 x 10^{-9} M/L) [33]. At about 800 μg of hefty daily doses, its accumulation in some tissues reaches ~ 3 x 10^{-8} M/L. Of course receptor density within the tissues is a major deciding factor in this case [1]. Like other PGs, Miso it shows inverted dose-response curve in many occasions [33 & 34]. As recorded previously, the generation of cAMP as a second messenger triggers the most biological responses [21].

The reported structure activity relationships of EP2 and EP4 receptors are approximately identical and in all situations both PGE1 /PGE2 act as the best agonist. In case of PGE1 and PGE2 binding, the respective K_i values for the EP receptors expressed in Chinese hamster Ovary Cells (CHO) are: EP1 = 20 / 36 nM, EP2 = 12 / 10 nM, EP3 = 1 / 1 nM and EP4 = 2 / 2 nM [1, 21]. The K_i values for Miso binding are: EP1 = 120 nM, EP2 = 250 nM, EP3 = 67 nM and EP4 = 67 nM whereas AH6809, an antagonist of PGE1 can specifically interact with the EP2 having the K_i = 350 nM. Apparently, the K_i for Miso's interaction with EP3 is seen lower (K_i = 67 nM) and closely comparable to EP4 that evidently shows its greater affinity toward EP3 than EP2. Regarding intracellular actions it is reported that besides the EP2, EP4 also raises the intracellular cAMP [21]. At this point the exact nature of EP receptor(s) expressed in W138 is unknown in addition to any of the associated biologic responses.

The rise of intracellular cAMP by Miso in human peripheral mono-nuclear leucocytes and other cell lines has been extensively studied and reported by the others [5, 6, 30, 35 & 36]. In this current study I have recorded that in human lung fibroblast cell line W138, Miso elevates cAMP in a dose-dependent way (EC_{50} ~ 7.5 μM) with same capacity both in ester or free acid form. The effect is blocked by specific PGE1 antagonist AH6809 indicating its interaction and above effect is carried via the EP2 subtype [21]. The cAMP elevation by Miso is heavily suppressed if W138 cells are pretreated with Colchicine whereas pretreatment with PTx enhances the effect but CTx somewhat lowers the generation. In case of FSK the effect is seen only to be additive. These incidents suggest that besides the EP2 there is a large possibility of involvement of EP3 subtype, especially the EP3α.

Materials and Methods

Materials: The commercially available Misoprostol was at first purchased from the Sigma Chemicals (10mg / vial, Catalog # M – 6806; Lot #.120K1160) either in free acid or methyl ester form (Catalog # M – 6807, Lot #. 120K1160). Afterward both forms of Miso and EP2 antagonist AH6809 were bought from Biomol (USA) (Catalog # PG – 052 & PG – 051) in 10 mg / vial. The

reagents were dissolved in high grade DMSO making a stock of 10 mg / ml which were kept in - 20 º C freezers for longer storing purposes. The other agents like, Forskolin (FSK), Isobutyl methyl xanthine (IBMX), Cholera (CTx) and Pertussis (PTx) toxins were procured from the Sigma and used after dissolving in DMSO making 1.0 mM solution as stock while storing in a refrigerator. The toxins were used fresh after solubilizing in Basal Eagle's Medium at required dilution just before the experiments.

Cell culture: The human lung cell line W138 was supplied by American Type Cell Culture (ATCC) and were cultured at 37ºC sterile incubator (5 % CO_2 & 95 % air) following the previous protocol with slight modifications using Basal Eagle's Medium (BMEM from Sigma) with 10 % Fetal Calf Serum (FCS from Hyclone, USA) and 1 nM EGF and 1 nM BFGF (Sigma, USA) [37]. The cell stock built up was later distributed after stripping with Trypsin (supplied in the cell culture kit) and allowed to grow in the same media in desired plates for experimental purposes. After each three passages, cells from fresh stocks were used. The fresh media were introduced in all occasions at each alternate day until reached to the confluent stage. Normally, after stripping the cells were plated at high density (~ 100,000 / well) in 24 well plates for their quick attaining to confluency which took five days. The fresh media were introduced in similar way at every alternate day. Each experiment was performed at identical confluent condition.

Experimental conditions for Miso treatment: Following our previously published procedure, the fresh media were always introduced the day before any experiment. Just prior to the beginning of experiment, media from overnight were replaced with pre-warmed (37ºC) serum free BEM (1ml / well) and further incubated for an hour (37ºC) inside the incubator [38]. After this hour long incubation the media were replaced again and fresh media (1 ml / well) having 1 µM IBMX (Phospho di- esterase inhibitor) were introduced and allowed to stand at same condition for 30 minutes. The use of IBMX was followed in each experiment. The dissolved Miso in DMSO were diluted in warm serum free BEM and added at 10 x concentrations at constant volume of 100 µl / well at different doses. The cells were incubated for an exact period of 20 minutes at 37ºC inside the incubator. At the end, equal volume (1 ml / well) of ice cold 10 % Tri-chloro Acetic Acid (TCA) was added to terminate the reaction. The plates were left frozen in acid media at - 20ºC freezers for future assay which were performed all at a time. The basal control was considered as without the Miso.

EP2 antagonist AH6809 treatment: The antagonist was added at 10 x concentrations dose-dependently in IBMX treated cells, afterward allowed to incubate for 10 minutes at 37ºC inside an incubator prior to the addition of Miso (100 µM). The cells were further incubated for 30 minutes

and later the reactions were terminated following the way as stated in previous section by adding 1ml of 10 % ice-cold TCA. The experiments were conducted four times (N = 4) with quadruplicate data points. The values at 100 μM Miso was accounted as a basal control.

Pertussis toxin (PTx) treatment: The confluent cells plated in 24 well plates were placed in fresh serum containing media for overnight. Next morning, the media was replaced with pre-warm (37ºC) serum free BEM (1 ml / well) and left for an hour at same incubating condition. Afterward, the media was replaced further with the same under similar condition. The experiment was designed as, untreated basal control, 100 μM Miso, PTx (4 μg / ml, final) and Miso + PTx. Prior to the addition of Misoprostol; the cells were at first incubated with PTx exactly for two hours. After adding the drug, incubation time was extended for a similar period of 30 minutes at 37ºC incubating temperature before terminating the reaction.

Cholera toxin (CTx) treatment: The above identical condition was maintained also in case of Cholera toxin. The CTx concentration used was 25 μg / ml, final.

Colchicine treatment: In case of Colchicine, similar experimental condition was also adopted. The final concentration of Colchicine was 100 μM, final.

Forskolin treatment: Similar media change was performed before initiation of the experiment except FSK (100 μM final) was added 2 minutes prior to the addition of Miso (100 μM final). The incubation time was followed exactly for 30minutes at 37ºC. The reaction order was basal control, FSK (100 μM final), Miso (100 μM final) and FSK + Miso.

cAMP Assay & data analysis: The assay was conducted using commercially available sensitive low pH immune assay kit purchased from R & D Systems Inc. (Minnepolis, MN). In all cases acetylation was conducted as per suggestion of the kit to achieve greater sensitivity of the assay. A separate standard curve was constructed for each plate. The plates were read at 405 nm wave length using a plate reader connected with the computer (Biorad, CA). The unknown data points were automatically evaluated by following the generated standard curve. In case of over the limit values, the samples were properly diluted to bring the values within the acceptable data range. Each data point was measured at three concentration range and in triplicate. The intra-assay coefficient for variability was always carefully accounted. Each experiments were conducted multiple times (N = 4 - 7) separately for raising the confidence level at least $p \leq 0.01$. Before starting the assay, all frozen plates were thawed at room temperature and the aliquots were at first exactly diluted to 1:500 folds which were then subjected to the assays following the direction. In all cases, the data analysis were conducted by using the software, INPLOT from GRAPHPAD Inc. (Sandiego, CA) and expressed with ± SEM for N = 4 experiments.

Results

cAMP Elevation by Misoprostol & Forskolin: The Fig – 2A shows the dose-dependent elevation of cAMP with EC_{50} = 7.4 ± 0.22 μM (N = 7, $p < 0.01$). Fig – 2B indicates that rise of cAMP level in case of ester which is very identical to the free acid $EC_{50} \sim 7.5$ μM. Interestingly, the dose-dependent addition of FSK also appears to be closely the same (EC_{50} = 9.1 ± 0.3 μM (N = 3, $p < 0.005$) (Fig – 2B).

Effect of EP2 receptor antagonist, AH6809: The EP2 receptor antagonist, AH6809 effectively and dose-dependently inhibits the cAMP elevation by Misoprostol (100 μM) with EC_{50} = 10 ± μM (N = 4). The observed inhibition is > 90 % (Fig – 3).

Effect of PTx and CTx treatment: The prior treatment with PTx enhances the cAMP elevation in presence of Miso by almost three fold. The toxin by itself has no effect on basal cAMP generation, Fig – 4A. On the other hand CTx behaves somewhat different way by lowering the effect of Miso stimulation at least by ~ 25 %; Fig – 4B.

Effect of colchicine and Forskolin treatment: Colchicine itself lowers the basal cAMP level. Interestingly it also suppresses the stimulated cAMP elevation by Miso (100 μM) about ≥ 90 % within doses ranging from 100 – 1.0 μM (d, e & f) in Fig – 4C. On the other hand, the effect of Forskolin is seen to be additive when presented along with the Miso (Fig – 4D).

Discussion

Miso is a widely known PGE1 analogue and as mentioned previously used mainly for the remedy of inflammation, ulcer formation or else inducing the labor [1 - 4]. Like its naturally available PGE1 counterpart, the pharmacological action of Miso is majorly mediated by the elevation of intracellular cAMP although PGE1 is known to produce other influences like increase of $[Ca^{+2}]_i\uparrow$ level via other EP receptor subtypes (EP1) as well [21 - 23]. Concerning the character of EP receptors like in case of EP2, which is linked to the G_s therefore its activation, translates to cAMP generation whereas for the EP3α, it is connected to G_i thus causing the inhibition during stimulated cAMP production [18]. In present study, using W138 cell line, Miso is seen to raise the intracellular cAMP level in a concentration dependent manner ($EC_{50} \sim 7\mu M$). Both free acid and its methylated ester act almost with equal potency (Fig – 2A). The value is closer to the dose-dependent generation by FSK ($EC_{50} \sim 10\mu M$) proving its powerful cAMP generating effect. Unlike previous reports, the equipotency of free acid ($-CO_2H$) and methyl ester ($-CO_2CH_3$) relates to the possible existence of high level of esterase on the cell surface. Presumably, the rapid de-esterification of methyl ester caused by that esterase in W138 releases the free acid of Miso in order to interact with its

respective receptor(s). Confirmatively, cAMP elevation occurs via the EP2 since AH6809, a specific PGE1 antagonist blocks the effect (~ 90 %) and it is also concentration dependent (EC_{50} ~ 10μM), Fig - 3.

It is somewhat noticeable that pretreatment with Pertussis toxin (PTx) elevates the cAMP level by Miso almost two fold (Fig – 4A). It is possible that the inactivation of G_i due to ribosylation by the PTx apparently helps enhance the effect of receptor associated G_s to promote cAMP generation further during agonist stimulation. The event indicates that a part of the receptor action of Miso is mediated via the G_i. It could be a possibility that the same receptor is coupled with both the G proteins G_s and G_i, which is a phenomenon often visualized during GPCR's overexpression or it could be, that in addition to EP2 Miso interacts with another subtype EP3α, which is linked to the G_i [18, 19, 39]. On the other hand, the behavior of CTx + Miso is seen to be different. CTx ribosylates the G_s which subsequently activates the enzyme, AC causing intracellular rise of cAMP [20]. The presence of Miso suppresses the event of cAMP rise by almost ~ 25 % signifying the ligand's capability to activate the G_i in W138 cells (Fig – 4B).

Previous works by many showed that Miso's action in producing cAMP is often synergized in presence of anti-mitotic drug Colchicine in number of cell lines [35]. In fact, this synergistic action is frequently exploited for treating many inflammatory purposes [35, 40 & 41]. Surprisingly in W138 cells the behavior is seen fully opposite. Here, colchicine suppressed the action of Miso by ~ 90 %. It is also noticed that by itself Colchicine lowered the basal cAMP level (Fig – 4C). It has been proven that Colchicine often activates AC by virtue of its ability to interact with the tubulin and disrupting the micro-tubule assembly [40 & 41]. It is postulated that this disruption relieves strains on part of the plasma membrane which in turn helps activation of AC by the G protein(s) during agonist interaction causing synergistic action, which is observed in the case of several agonist – receptor interactions [40 - 42]. Relying on similar argument but in a reverse mode, it can be hypothesized also that in W138 cells, the disruption of micro-tubule might help release the G_i enabling to suppress the action of AC during Miso-receptor interaction more actively. The effect lowers the cAMP generation to a considerable extent (Fig – 4C).

The FSK is known to activate AC directly causing the rise of is cAMP. In W138 cells, Miso along with the FSK produced only an additive effect (Fig – 4D). It is therefore logical to presume that in the event of receptor mediated activation, G_s plays a separate and independent role in addition to the direct action of FSK. Therefore together they only show the additive result. In that course, the role of G_i appears insignificant and also not viewed during cAMP generation by the CTx

which is partially suppressed by the Miso possibly by the action of G_i (Fig – 4B & 4D). It is a possibility that direct action of FSK can overrule the role of G_i.

Conclusion

The dose-dependent intracellular rise of cAMP in human lung fibroblast W138 by Misoprostol occurs through the interaction of EP2 receptor ($EC_{50} \sim 7$ μM). In addition to EP2, Miso possibly interacts with the other category EP3α, since PTx treatment enhances the cAMP response of the drug by two fold. On the other hand CTx produces an opposite effect in its presence by lowering the level (25%) of cAMP. Disruption of tubulin assembly by Colchicine overrules the Miso's action considerably (> 90 %).

Acknowledgement

Author gratefully acknowledges the support from Sepracor Inc. (Marlboro, MA) along with the immense help provided by the esteemed colleague, Drs. Steve Jones & Yael Schwartz for showing much of their interest during carrying out this work. Much of the gratitude also goes to Dr Rajesh Barur, University of Massachusetts Medical School at Worcester for his kind help in various ways.

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Figure legend

Figure- I. Chemical structure of various PGs, Misoprostol and the EP2 antagonist, AH6809.

Figure – 2. Dose-dependent cAMP elevation by free acid and methyl ester of Miso in W138 cell line. The calculated EC_{50} = 7.4 ± 0.22 μM (N = 7 Expt, p ~ 0.01). Almost similar elevation is observed in case of FSK (EC_{50} = 10.12 ± 0.06 μM (N = 5 Expt, p ~ 0.006).

Figure – 3. Dose-dependent lowering of cAMP elevation by AH6809 when stimulated in presence of 100 μM Miso. The EC_{50} = 10.04 ± 0.10 μM (N= 4 Expt; p ~ 0.008). The basal cAMP level = 0.096 ± 0.009 nMol /ml whereas in presence of 100μM Miso = 2.54 ± 0.25 nMol/ml. Similar set of experiments and p values are ~ 0.005 to 0.01.

Figure – 4.

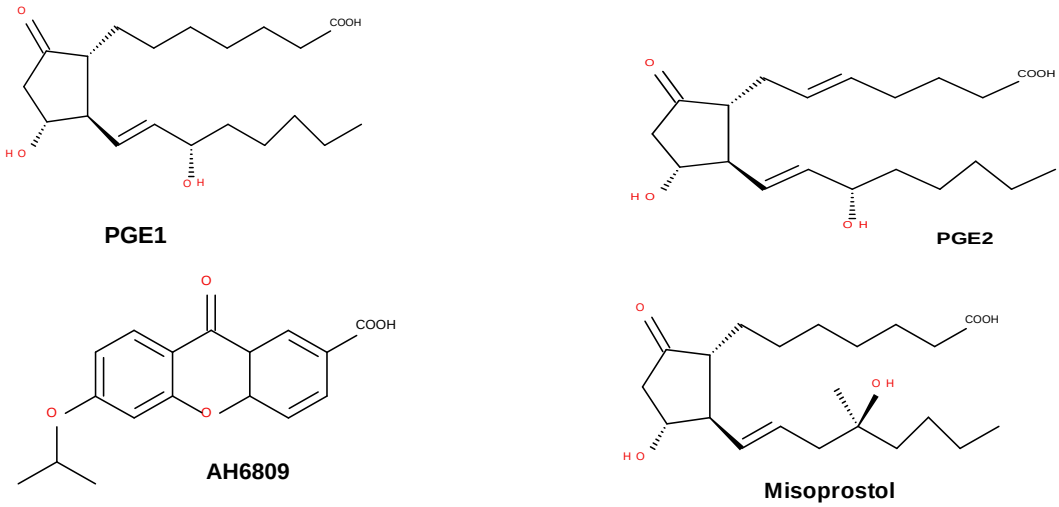
A); The % of change in cAMP level while pretreating the W138 cells with PTx (4μg/ml) before the addition of Miso (100μM). N = 4 Expt; p ~ 0.002 – 0.005.

B); Effect of cAMP level during similar treatment with CTx (25μg/ml) and Miso (100 μM). N = 4 Expt, p ~ 0.004 – 0.008.

C); The lowering of cAMP level by Colchicine and its combine efficiency in presence of Miso (10μM).a) – basal level, b) 10μM Miso alone, c) 100 μM Colchicine, d) 10μM Miso + 100μM Colchicine, e) 1.0 μM Miso + 10 μM Colchicine, f) 0.1 μM Miso + 1.0 μM Colchicine. The significances are tested by ANOVA, p ~ 0.003 - 0.007.

D); The combined effect of FSK (1μM) and Miso (10 μM) on cAMP level in W138 cell line. N= 3 Expt. P ~ 0.004 – 0.01.

Figures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Misoprostol

Figure-I

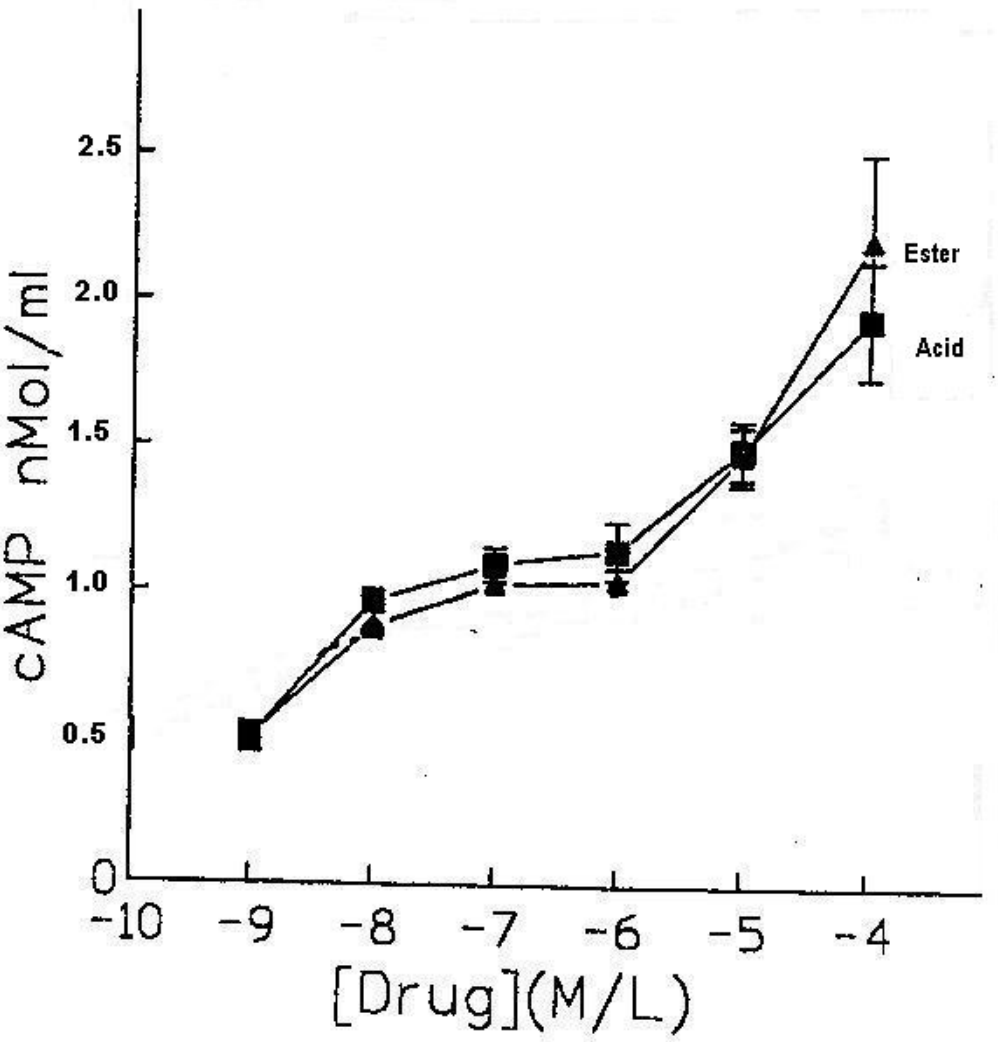

Figure – 2

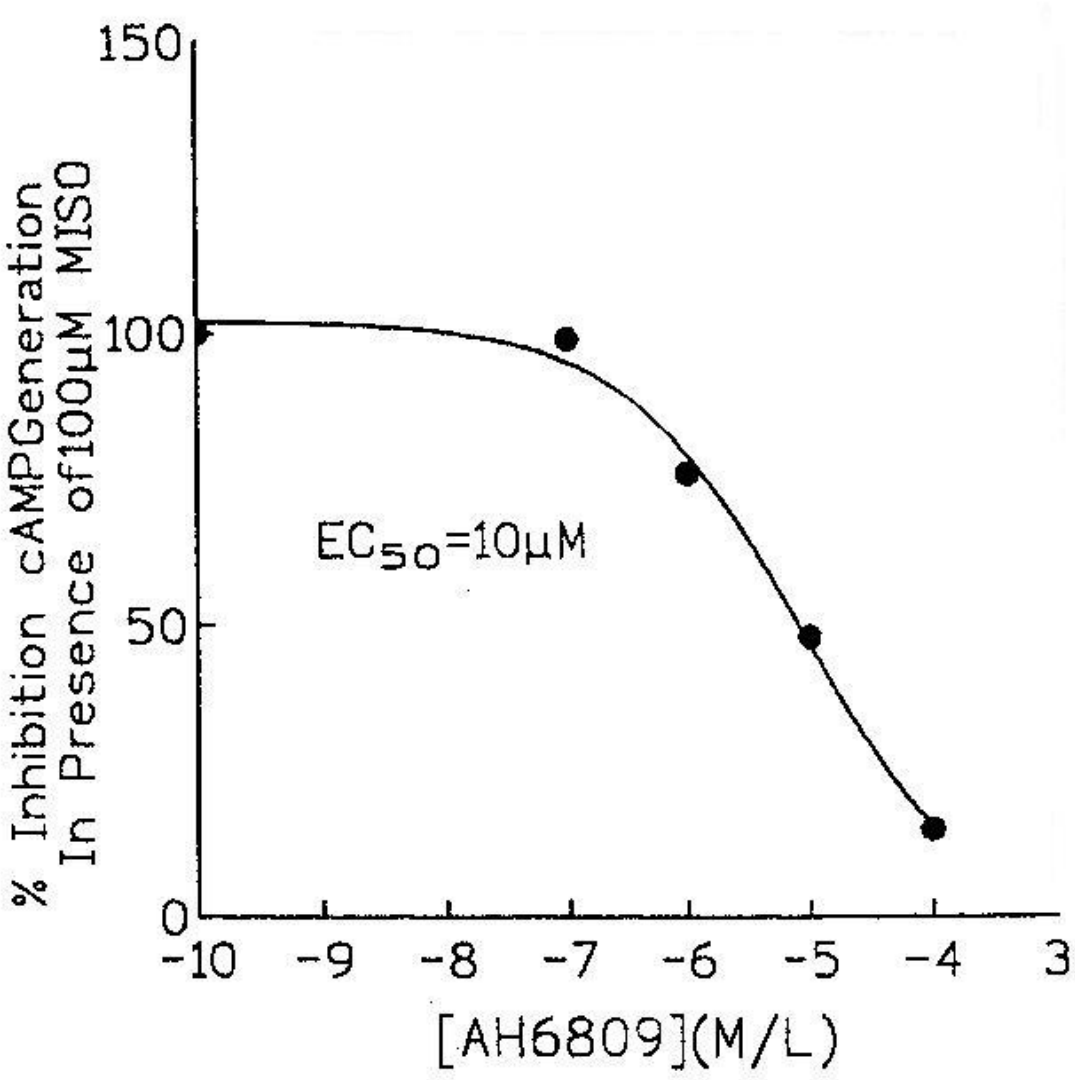
150
100
50
0
% Inhibition cAMPGeneration
In Presence of100μM MISO
EC50=10μM
-10
-9
-8
-7
-6
-5
-4
3
[AH6809](M/L)

Figure – 3

FF

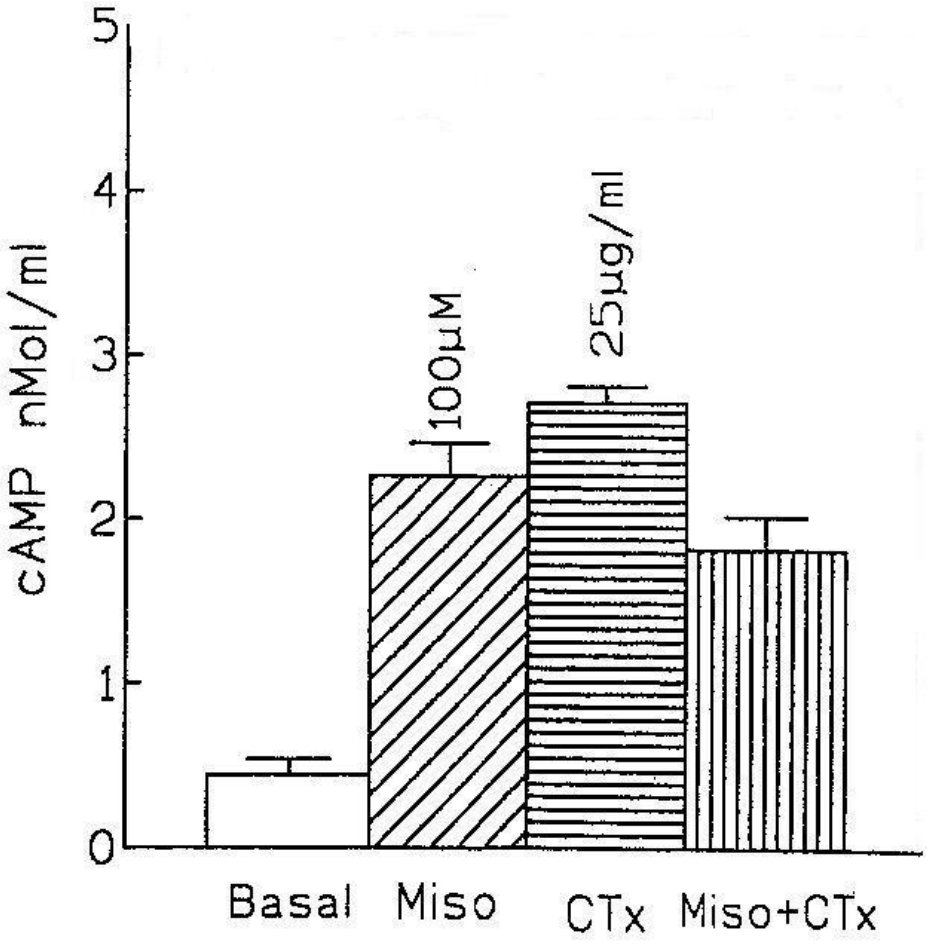

Figure – 4B

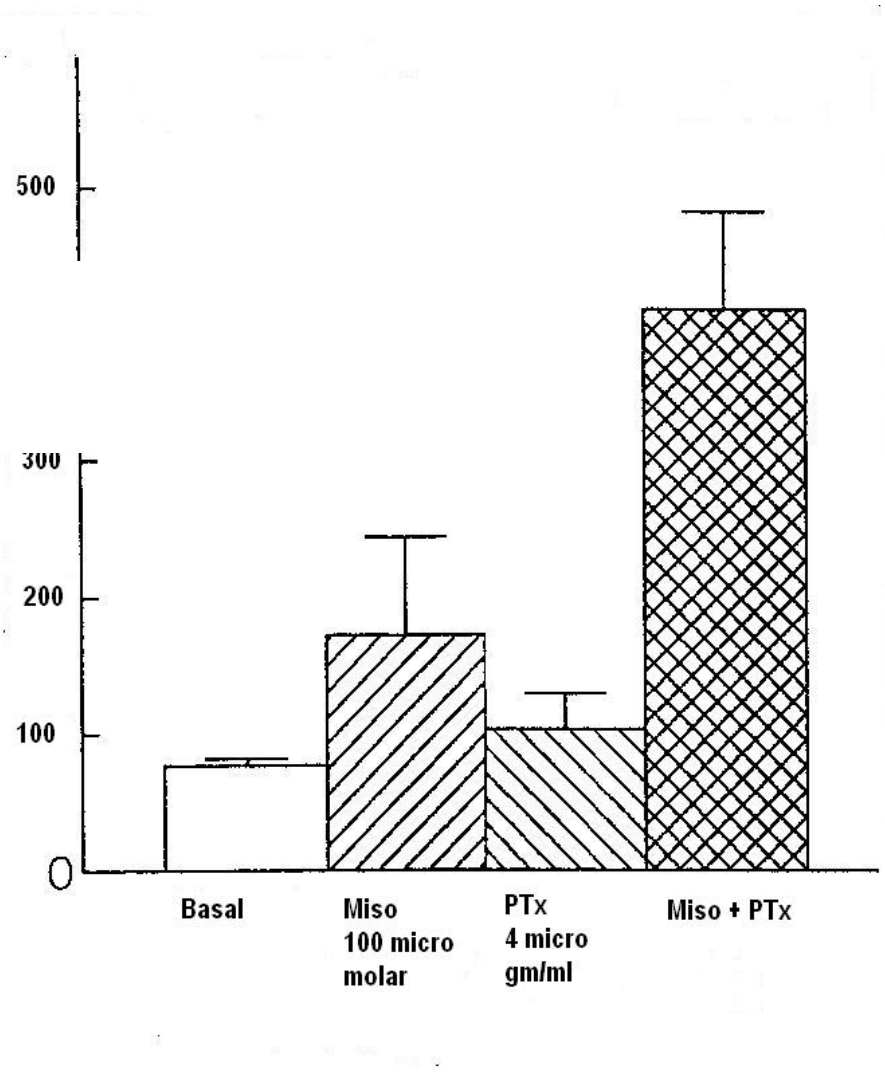
500
300
200
100
0
Basal
Miso
100 micro
molar
PTx
4 micro
gm/ml
Miso + PTx

Figure – 4A

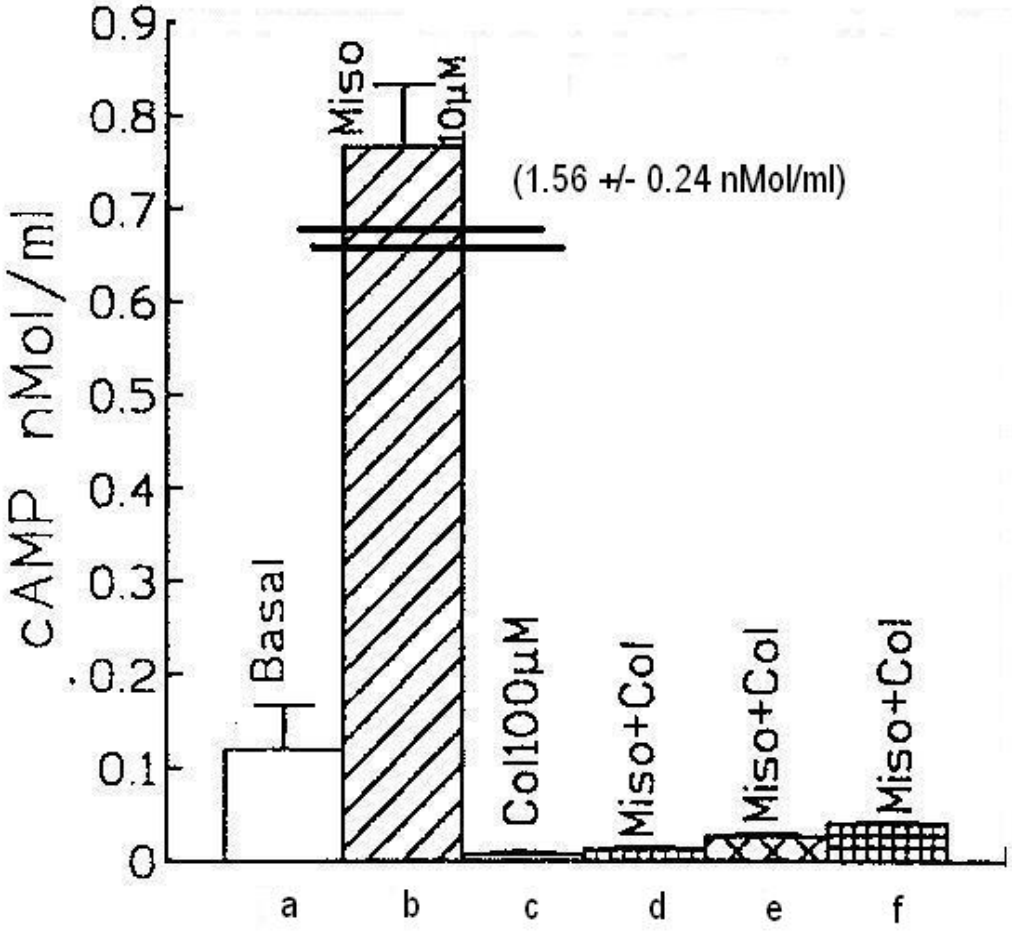
cAMP nMol/ml
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Miso
10µM
(1.56 +/- 0.24 nMol/ml)
Basal
Col100µM
Miso+Col
Miso+Col
Miso+Col
a
b
c
d
e
f

Figure – 4C

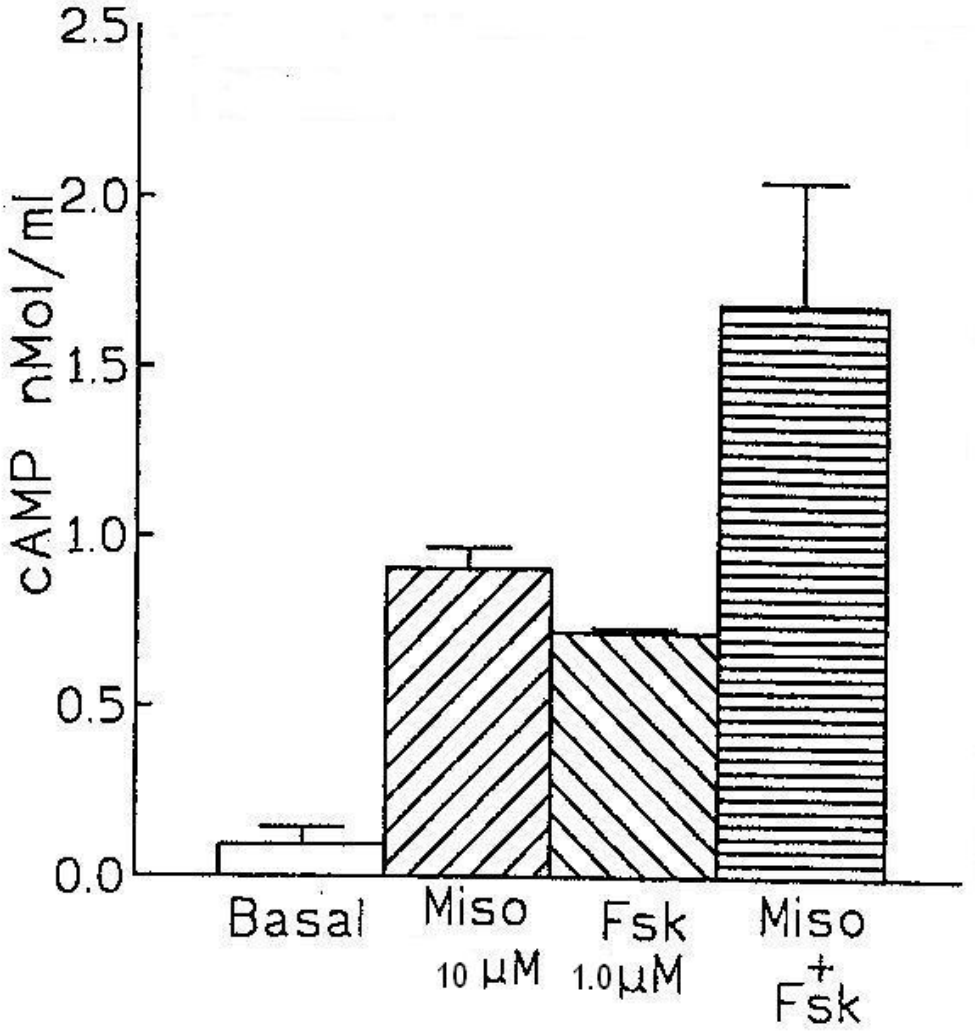

Figure – 4D