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Inhibition of human myometrial cyclic AMP phosphodiesterase by uterine relaxant drugs

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The relaxation of the uterus induced with β-adrenergic agents, by an increased cAMP level following an adenylate cyclase stimulation, could also be obtained with several drugs or hormones which enhance the cAMP level through a cAMP phosphodiesterase (cAMP PDE) inhibition [1, 2]. In 1968, Dousa and Rychlik reported that cAMP PDE is present mainly in the cytosolic fraction from rat myometrium but till now a few studies have been made and the knowledge about cAMP PDE in the human myometrium is almost nil [3].

The purpose of our work was to determine the kinetic properties of cAMP PDE in homogenates of myometrial tissue taken from human pregnant uterus and then to investigate the effects of steroids and of several pharmacological agents used to modify uterine motility.

8[^{3}H] cyclic 3′,5′-adenosine-monophosphate (spec. act. 27 Ci/m-mole) and ^{14}C adenosine (spec. act. 385 mCi/m-mole) were supplied by CEA Saclay, France. Adenosine 3′,5′-cyclic monophosphate acid (cAMP), adenosine 5′ monophosphoric acid (5′-AMP), imidazole, snake venom (Crotalus atrox), and 20 β-dihydroprogesterone (20β-hydroxy-Δ^4-pregnen-3-one) were supplied by Sigma Chemical Co. Progesterone, Estradiol-17β and Estrone were gifts from Laboratoires Roussel-Uclaf. Estrone-3 methyl ether, Estradiol-17β-3 methyl ether, diethylstilbestrol (3,4-*Bis* (4 hydroxyphenyl-3-hexane)) were purchased from Steraloïds.

Anion exchange resin (AG 1-X 2 200–400 mesh) was obtained from Bio-Rad. All the solvents (analytical grade) were from Merck and other chemicals and drugs from Calbiochem. Indomethacin and flufenamic acid were generously provided respectively by Merck Sharp Dohme and Parke Davis Labs.

Myometrial tissue was obtained from full-term pregnant women without endocrine disorders, during elective caesarian section before onset of labour. A piece of 150–200 mg of tissue was excised in the uterine body near the placental insertion and was carefully freed of endometrium. The muscle sections were collected on ice, cut into small fragments and homogenized (200 mg/6 ml) with a ground-glass Potter–Elvehjem apparatus in 0.25 M sucrose, 1×10^{-2} M Tris–HCl (pH 8).

Freezing the homogenate at − 20° was not followed by any detectable loss of enzymatic activity for at least 2 months. cAMP phosphodiesterase activity was measured by the two steps isotopic procedure according to Thompson and Appleman [4] as described previously [5]. The reaction mixture contained generally 8×10^{-2} M Tris–HCl (pH 8), 5×10^{-3} M magnesium acetate, 1.5 μCi/ml [^{3}H]cAMP, unlabelled cAMP at various concentrations and enzymatic preparation in a final volume of 0.200 ml. Steroids were dissolved in 10 μl of 100% ethanol. It was controlled that ethanol did not have any effect on cAMP phosphodiesterase and 5′ nucleotidase activities. In addition it was verified that drugs and hormones tested did not modify 5′ nucleotidase activity in our experimental conditions. All the assays were carried out in conditions of linearity with respect to time and protein concentration, allowing measurements of the initial rates of the reaction. Protein concentrations were determined by the method of Lowry *et al.* using bovine serum albumin as standard [6].

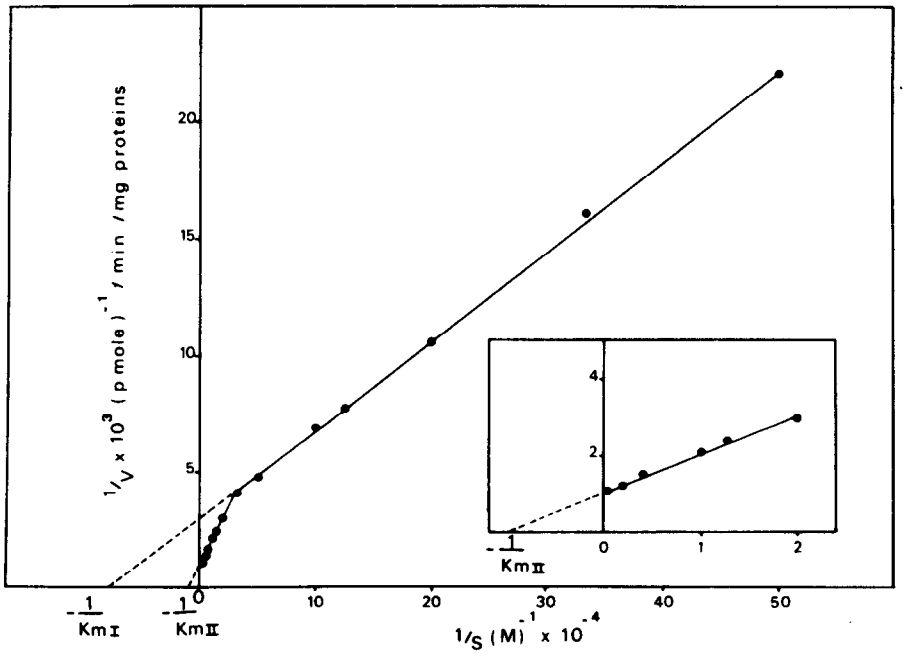

Fig. 1. Kinetic data for PDE in human myometrial homogenate with respect to cAMP concentration. The conditions were the same as described in the text. Data at high substrate concentrations (left portion of the graph) were replotted on an expanded scale.

As shown in Fig. 1, kinetic analysis of cAMP PDE activity vs cAMP concentration by double reciprocal plots according to Lineweaver–Burk reveals two mathematically identifiable enzymatic activities. In the range of cAMP concentration from 1×10^{-7} M to 2×10^{-5} M an apparent Michaelis constant value: K_m 1: $1.26 \pm 0.14 \times 10^{-5}$ M and a V_{max} value of 457 ± 205 pmoles/min/mg of proteins are obtained corresponding to the high affinity form. In the range of 2×10^{-5} M to 8×10^{-4} M a second series of constants is determined revealing a form of enzymatic activity having a lower affinity: K_m 2: $0.91 \pm 0.17 \times 10^{-4}$ M, $V_{max} = 1006 \pm 147$ pmoles/min/mg of proteins. As in numerous other mammalian tissues, multiple forms of cAMP PDE activity have been recently reported in rat uterine homogenate and in myometrial 1000 *g* supernatant from pregnant rhesus monkey [7, 8]. In agreement with these latter studies, we show in this report that, at high substrate concentration, the human high affinity enzyme contributes significantly to the kinetic behaviour of the low affinity form.

Human myometrial cAMP PDE requires Mg^{2+} to express its maximal activity which is achieved between 3×10^{-3} M to 5×10^{-3} M of this cation. This requirement is identical at low (8×10^{-6} M cAMP) and high (4×10^{-4} M cAMP) substrate concentrations. We noticed that 30–40 per cent of the maximal activity is present without any Mg^{2+} addition. This can be explained by the fact that the endogenous concentration of this cation determined by atomic absorption method is 0.165 ± 0.045 mg/g of wet tissue which corresponds approximately to 3×10^{-4} M in our incubation mixtures. Imidazole at 10^{-2} M induces a 35 per cent stimulation of activity for the low affinity form whereas the high affinity form is unaffected.

Several nucleotides appear to be inhibitors at high concentrations: 1.5×10^{-3} M GTP, 9.8×10^{-4} M adenosine and 5.5×10^{-4} M cGMP induce a 50 per cent inhibition of the high affinity form. At 1×10^{-3} M the 5'AMP has no effect. Among all nucleotides tested, cGMP is the most effective. The inhibition of the high affinity form was found to be competitive in a range of concentrations of cGMP lower than 5×10^{-4} M, becoming more complex at higher concentrations.

The two forms of the enzyme are competitively inhibited by theophylline and non competitively by caffeine. In contrast papaverine shows a complex type of inhibition which is competitive at low substrate concentrations and of mixed type at high substrate concentrations. Indomethacin and flufenamic acid inhibit cAMP PDE activity to the same extent. In high affinity conditions (8×10^{-6} M cAMP) both drugs at 7×10^{-4} M induce nearly 50 per cent inhibition. As with the other inhibitory agents tested, their effects occur preferentially on the high affinity form which is competitively inhibited by indomethacin, progesterone and diethylstilbestrol and could then be considered as involved in the hormonal regulation of the cAMP level (Table 1). In order to ascertain the potential physiological significance of the hormone effects, also found in some steroidogenic tissues [5, 9] other steroids and derivatives: 20 β-dihydroprogesterone, estradiol-17 β, estradiol-17 β-3-methyl ether, estrone, estrone-3 methyl ether, estriol and estriol-3 methyl ether were tested at 6×10^{-4} M concentration. They show negligible or no inhibition.

Some inhibitors tested on this human myometrial enzyme are known to modulate uterine motility [10]. Theophylline, given intravenously, reduces the uterine contractility of pregnant women [11]. The observed correlation obtained with papaverine, between the relaxation of the rat uterus and the increase in cAMP concentration, seems to be due to cAMP PDE inhibition [12]. Indomethacin is a potent and useful drug in the treatment of premature labour in human. However in this case it has been recently suggested in pregnant rhesus monkey that cAMP contributes only partly to induce relaxation [8]. Indeed, nonsteroidal anti-inflammatory drugs are known to inhibit prostaglandin synthetase activity at much lower concentrations than the K_i for PDE [13, 14]. *In vivo* and *in vitro* experiments demonstrated the inhibitory effect of progesterone on the human uterine contractility [15, 16]. However, the endogenous level of this steroid is much lower than the concentration needed to elicit the cAMP PDE inhibition *in vitro* and the physiological significance of such an action remains difficult to ascertain. Diethylstilbestrol which is ten times more potent than progesterone to inhibit cAMP PDE was administered to pregnant women with the purpose to facilitate the uterine development but this compound was devoid of any effect on uterine contractility. One explanation to their different effects could be the cAMP partition within the intact cell into several pools acting on separate biochemical mechanisms.

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Table 1. Inhibition by some drugs and hormones of myometrial cAMP PDE activity in high and low affinity conditions. Each K_i value is the mean of duplicate experiments, made with two different concentrations of inhibitor, on three different myometrium

Inhibitors	Low K_m conditions 2×10^{-6} M to 2×10^{-5} M cAMP	High K_m conditions 5×10^{-5} M to 2×10^{-4} M cAMP
Theophylline	Competitive type K_i: 0.9×10^{-3} M	K_i: 2.6×10^{-3} M
Caffeine	Non competitive type K_i: 2.8×10^{-3} M	K_i: 7.0×10^{-3} M
Papaverine	Competitive type K_i: 3.9×10^{-6} M	Mixed type
Indomethacin	Competitive type K_i: 3.4×10^{-4} M	
Progesterone	Competitive type K_i: 6.0×10^{-4} M	
Diethylstilboestrol	Competitive type K_i: 7.2×10^{-5} M	

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Partition coefficients of alkanols in lipid bilayer/water

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Long chain alcohols induce narcosis and anesthesia in a variety of animals. Like other anesthetics they also modulate a variety of other functions in biomembranes and in lipid bilayers: these include expansion [1], motional and phase properties [2–7], permeability [8] and functions of membrane-bound enzymes [9, 10]. Although the molecular mechanisms involved in the perturbation of any of these functions are not established, it is generally accepted that alcohol molecules localized in the membrane are responsible for such effects. Thus, a correlation is observed between the *n*-octanol/water partition coefficients of a series of *n*-alkanols and their equipotency concentrations for induction of a variety of membrane phenomena. Such correlations suggest that the incremental free energy for the incorporation of methylene residues in *n*-octanol and in membrane systems is similar [11]. Such correlations, however, do not give any information about the actual amount of alcohol partitioned into the membrane phase. In this communication, we report partition coefficients in lecithin bilayer/water for homologous alkanols.

Partition coefficients were determined by measuring the concentrations of alkanols in the aqueous phase in the presence (C_w) and in the absence (C_l) of liposomes. The amount of alcohol incorporated into the lipid phase (A_l) was obtained by difference. Thus $A_l = (C_l - C_w)V_w$, and the concentration of alcohol in the membrane phase $C_l = A_l/W_l$ where W_l is the weight of lipid in V_w g of water. Thus, the partition coefficient, the ratio of the concentrations of alcohol in the two phases, $P = C_l/C_w = (C_l - C_w)V_w/C_w \cdot W_l$. It may be noted that the partition coefficient is defined as: g solute/g of lipid phase divided by g solute/g of aqueous phase. It is dimensionless. As an approximation, the amount of the lipid phase was taken to be the same as the amount of lipid present in the mixture. Experimentally, 200 μl of an aqueous solution of alcohol was mixed with 100 μl of hand-shaken liposomes. The final lipid concentrations in each tube was either 0 (control), 10, 20 or 50 mg/ml. The tubes were allowed to equilibrate at room temperature (24°) for 30 min and then centrifuged for 3 min at 12000 *g*. Separate experiments showed that equilibrium was complete in this period, since no significant change in the alcohol concentration was noted even after 24 hr of incubation. About 50 μl of the supernatant fraction was withdrawn, in which the amount of alcohol was determined by gas chromatography. Five-to 10-μl samples of the aqueous phase were injected into a 6 ft (3/16 in. i.d.) column packed with Porapak Q (Supelco, Inc., Bellefonte, PA., U.S.A.) on a model 5750 Hewlett Packard gas chromatograph equipped with a flame ionization detector. The column, port and detector temperatures were adjusted and programmed to optimize linearity between the volume of the alcohol solution injected and the area of the elution profile peak. The amount of each alcohol could thus be detected with an accuracy of better than ± 5 per cent. The value of a partition coefficient was calculated from the mean of the values for three samples, with three injections from each sample. There are several possible sources of error in these measurements [12]. The range of concentrations involved, the design of the experimental setup and the magnitudes of the partition coefficients are such that, with the controls employed, the overall error in a measured partition coefficient is less than ± 10 per cent or ± 2 (whichever is larger) of the absolute partition coefficient. This method gives a considerably larger margin of error if the partition coefficients are lower than 1 or larger than 2000.

The ratio of each concentration of alcohol in lipid (obtained by difference) to the concentration in water—that is, the measured partition coefficient for each alkanol, is presented in Table 1. The following features of these data may be noted.

Partition coefficients increase monotonically with chain length. The incremental free energy for the partitioning of a methylene residue calculated from the data is − 745 and − 630 cal/mole for the dipalmitoyl and egg lecithin liposomes respectively. Similarly, it takes 2.81 kcal/mole and 1.51 kcal/mole to partition the hydroxyl group in a dipalmitoyl and egg lecithin bilayer. These values compare favorably with the values observed for the partition coefficients of alcohols in isotropic organic solvents/water systems (column 5; [13–15]) and other membrane/water systems (column 6).

The partition coefficients for dipalmitoyl lecithin liposomes are smaller than the corresponding values for egg lecithin liposomes. However, this difference decreases with increasing chain length. Although alkanols incorporated into a bilayer induce gel to liquid crystalline phase transition, the dipalmitoyl lecithin bilayer, at the concentration employed in this study, would be exclusively in the gel phase at room temperature [16]. This is also supported