Studies on Theophylline Induced Activation of Glycogen Phosphorylase in Rat Uteri[1] (36672)

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Theophylline in sufficiently high concentrations (> 1 m*M*) inhibits motility in the isolated rat uterus (1) and in lower concentrations, it potentiates the inhibitory effects of catecholamines (2) and of cyclic adenosine 3′,5′-monophosphate (C-AMP) on motility (1). Since catecholamines increase phosphorylase *a* activity in addition to inhibiting contractions in the rat uterus (3) experiments were made to determine whether theophylline alone would increase the activity of this enzyme and whether there is an interaction with the phosphorylase activating hormones estradiol, epinephrine and oxytocin (3). The results show that estradiol and epinephrine but not oxytocin, modify the effect of theophylline quantitatively in increasing phosphorylase *a* activity in the uterus (myometrium).

Methods. Adult Long-Evans rats were ovariectomized and 7 days later were injected with 50 μg estradiol benzoate, sc. After 48 hr, the rats were anesthetized with sodium pentobarbital (60 mg/kg, ip) and the uterine horns were removed and trimmed separately. Uteri from rats ovariectomized for 9 days and without estrogen treatment were also used. The horns of each uterus were suspended by means of hooks and weights in separate chambers containing Krebs–Ringer bicarbonate medium with added glucose (11 m*M* concentration) (pH 7.3) at 37° and gassed with 95% O_2:5% CO_2, (4). Each chamber with a capacity of 33 ml accommodated 3 horns and 1 horn of each rat served as a control in a separate chamber. The procedure usually followed was to preincubate the horns for 45 min to allow the elevated phosphorylase *a* activity which resulted from manipulating the tissue in preparation for the bath, to subside to resting levels. The uterine horns were either transferred to a fresh bath containing test substances or the latter were added to the bath directly. After treatment, the horns were quickly frozen between two smooth blocks of solid CO_2 before the hooks and weights were cut free. Each horn was sealed in parafilm and stored at −90° until assayed for phosphorylase.

Theophylline in the amounts used did not alter the pH of the bath. It was observed that aminophylline was as effective as theophylline at half the concentration of theophylline but had to be neutralized before using. The following were used: *l*-epinephrine hydrochloride and oxytocin (Parke Davis Co), reserpine (Serapsil, Ciba), C-AMP as the dibutyryl derivative, DB-C-AMP (Boerhinger-Mannheim).

The assay methods for phosphorylase *a* activity (without added adenylic acid) and total phosphorylase activity (with adenylic acid) were previously reported (5). Phosphorylase *a* is given as micromoles P_i liberated from glucose-1-PO_4 by 100 mg tissue in 30 min at 37° and as a percentage relative to the total enzyme activity ± SE of the mean.

Results. It was necessary to determine the concentration of theophylline and the time of exposure of estrogen-primed uteri which would increase phosphorylase *a* activity. A concentration of 5×10^{-3} *M* which was about the limit of solubility in Krebs–Ringer medium was chosen. Immediately following removal from the rats the uteri were suspended in a bath containing 5×10^{-3} *M* theophylline and after different intervals were as-

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sayed for enzyme activity. After 20 min exposure to theophylline, the level of phosphorylase *a* activity was significantly higher than in the control uterine horn. After 45 min exposure, the absolute level of phosphorylase *a* activity was only slightly higher than that at 20 min while the control level was slightly lower. Subsequently, rat uteri were preincubated in the medium for 45 min to allow the enzyme activity to subside to base levels before treating with theophylline for 20 min.

Phosphorylase *a* activity in preincubated uteri treated with 5×10^{-3} *M* theophylline for 20 min was significantly higher than in the controls (Table I). When treatment was for 45 min, the net increase in enzyme activity above control levels was greater than that observed at 20 min but the absolute level of activity was only slightly higher. In a similar experiment, the same concentration of theophylline in contact with uteri for 5 and 10 min was without effect on the enzyme activity (data not shown). Concentrations of 5×10^{-4} and 5×10^{-5} *M* theophylline acting for 20 min did not increase the enzyme activity (Table I).

TABLE I. Phosphorylase *a* Activity in Estrogen Stimulated Rat Uteri Induced by Theophylline *in Vitro*.[a]

No. of rats	Theophylline *M* concn	Phosphorylase *a* (μmoles Pi)	Activity ratio ($a/t \times 100$)
5	5×10^{-5}	5.4 ± 0.7	28.3 ± 2.2
	None	5.8 ± 0.4	29.7 ± 1.3
5	5×10^{-4}	4.9 ± 0.3	24.5 ± 0.9
	None	5.8 ± 0.1	29.7 ± 0.8
5	5×10^{-3}	10.4 ± 0.9[b]	47.6 ± 2.1[b]
	None	6.3 ± 0.4	31.0 ± 2.7
6[c]	5×10^{-3}	11.3 ± 0.6[b]	52.5 ± 1.8[b]
	None	4.0 ± 0.3	22.6 ± 1.2
6[d]	5×10^{-3}	7.0 ± 0.1	35.7 ± 1.2[b]
	None	5.2 ± 1.0	25.8 ± 2.7

[a] Uteri preincubated 45 min and transferred to medium containing theophylline for 20 min except as indicated.

[b] Differences from respective controls significant, $p < .01$, other differences not significant.

[c] Uteri incubated with theophylline 45 min.

[d] Uteri from ovariectomized rats not treated with estrogen.

Following exposure of ovariectomized rat uteri to 5×10^{-3} *M* theophylline for 20 min, phosphorylase *a* activity increased but was significant only if the results were expressed as a percent of total activity (Table I). The net increase in activation above the respective controls was less than that in estrogen-primed uteri. It was noted that with this concentration of theophylline, uterine horns from ovariectomized rats and those estrogen-primed relaxed and showed no spontaneous contractions. At a concentration of 5×10^{-4} *M*, only one of 5 uterine horns gave slight contractions and at 5×10^{-5} *M*, all horns showed feeble spontaneous contractions.

Experiments were designed to determine whether theophylline would affect previously elevated levels of phosphorylase *a* activity induced by oxytocin and epinephrine. Estrogen-primed uteri after preincubation were stimulated with oxytocin (0.1 U/ml) and with epinephrine (3.3 μg/ml) for 10 and 5 min, respectively. In the first experiment, all uterine horns in the separate chambers were treated with oxytocin. One group was then washed and placed in a chamber containing 5×10^{-3} *M* theophylline for 20 min and the control group were placed in Krebs–Ringer medium for the same period. In the second experiment, only one group of horns were treated with oxytocin and then both groups were transferred into theophylline for 20 min. The same experimental design was used for epinephrine.

After an initial stimulation with oxytocin followed by theophylline, uterine phosphorylase *a* activity was significantly higher than in the controls which were exposed to oxytocin, then to Krebs–Ringer (Table II). In the second experiment, however, the enzyme activity was elevated in the presence of theophylline to the same level, whether or not the uteri were pretreated with oxytocin. Phosphorylase *a* activity in uteri pretreated with epinephrine followed by theophylline was very high compared to the low activity in the horns which were placed in Krebs–Ringer after epinephrine treatment. However, enzyme activity in uteri stimulated sequentially

TABLE II. Effect of Theophylline on Phosphorylase *a* Activity Levels in Isolated Rat Uteri after Initial Activation with Oxytocin and Epinephrine.

Initial treatment[a]	Time (min)	Second treatment for 20 min	Phosphorylase *a* (μmoles P_i)	Activity ratio ($a/t \times 100$)
Oxyto.	10	Theophylline	8.0 ± 0.6	39.2 ± 0.5
	10	Krebs–Ringer	6.1 ± 0.5	30.1 ± 2.2
	10	Theophylline	8.3 ± 0.3[b]	36.9 ± 1.5[b]
None	10	Theophylline	8.0 ± 0.4	39.7 ± 0.8
Epin.	5	Theophylline	10.5 ± 0.6	51.5 ± 1.9
	5	Krebs–Ringer	5.6 ± 0.6	28.1 ± 1.7
	5	Theophylline	11.6 ± 0.9	53.1 ± 1.9
None	5	Theophylline	8.2 ± 0.7	42.3 ± 2.3

[a] Six uterine horns per treatment.

[b] Difference of means within experiment, not significant; other differences are significant, $p < .05$ or less.

with epinephrine and theophylline was also significantly higher than that observed with theophylline stimulation alone (Table II). It was previously noted that elevated levels of phosphorylase *a* activity in uteri stimulated with these same concentrations of oxytocin for 10 min and epinephrine for 5 min will return to base control levels when placed in Krebs–Ringer within 10 and 5 min, respectively (5). The uteri remained contracted in the presence of oxytocin and were relaxed with epinephrine and theophylline.

It was considered possible that residual catecholamines in the uteri might be in part responsible for the effects of theophylline on phosphorylase activity and that by lowering the catecholamine content, the effects of theophylline would be depressed. Since reserpine was reported to lower catecholamines in the rat uterus (6), a group of estrogen-primed and of ovariectomized rats were adrenal-demedullated and treated with reserpine. Demedullation was performed at 9 AM and injection with reserpine (2.5 mg/kg, sc) made at 3 PM. Controls were sham-operated only. At 9 AM the following day, isolated uteri from experimental and controls were treated with theophylline. Phosphorylase *a* activity was the same in the theophylline treated uteri from demedullated-reserpinized rats and their respective controls in both groups (Table III). In another experiment, 3 ovariectomized rats were injected with reserpine (2.5 mg/kg, sc) on 2 successive days and 3 controls were untreated. On the third day, all uteri were isolated and stimulated with theophylline. Phosphorylase *a* activity expressed as a percentage of total activity was 37.1 ± 1.8 for the reserpinized rats and 40.6 ± 0.6 for the controls.

Catecholamines stimulate adenyl cyclase to produce C-AMP in the rat uterus (7), and presumably as in certain other tissues, C-AMP mediates the catecholamine induced increase in phosphorylase *a* activity (8). To determine whether C-AMP would increase

TABLE III. Uterine Phosphorylase *a* Activity Induced by Theophylline *in Vitro*: Uteri from Reserpine-Treated Adrenal-Demedullated Rats vs Controls.

Treatments[a] of rats	Phosphorylase *a* (μmoles P_i)	Activity[b] ratio ($a/t \times 100$)
Estradiol, demedul., reserpine	5.6 ± 0.4	32.2 ± 1.4
Estradiol	5.0 ± 0.5	32.8 ± 2.4
Ovariectomy, demedul., reserpine	3.9 ± 0.3	21.6 ± 0.9
Ovariectomy	5.2 ± 0.5	26.2 ± 2.1

[a] All uteri stimulated with 5×10^{-3} *M* theophylline for 20 min.

[b] Difference of means in each experiment, not significant; 4 rats in each treatment.

phosphorylase activity *in vitro*, isolated uteri from 4 rats ovariectomized for 2 weeks and estrogen-primed and also from 4 ovariectomized rats were treated with dibutyryl-C-AMP (1×10^{-3} *M*) for 10 min. Phosphorylase *a* as a percentage of total activity in 3 of 4 stimulated horns was 32.6, 23.4, and 29.4 and in the respective controls it was 26.5, 15.4, and 22.4. One horn failed to respond. DB-C-AMP failed to stimulate the enzyme activity in all uterine horns from ovariectomized rats, the activity as a percentage of total was 15.3 ± 1.1 in treated horns and 16.5 ± 1.8 in the controls. Upon addition of DB-C-AMP, all uteri gave one or two contractions immediately, then all motility ceased thereafter.

The experiments on the effects of theophylline were made *in vitro* in order to obtain additional observations on contractile behavior of the uterus and to circumvent difficulties arising from the necessary freezing of the uteri *in situ* when studies are made *in vivo*. When estogen-primed rats were injected with theophylline (90 mg/kg, ip) and the uteri were removed 50 min later, the percentage of phosphorylase *a* activity relative to total activity was 41.4 ± 4.4 ($n = 5$) and in the controls, 37.1 ± 1.2 ($n = 5$). However, injection of the readily soluble aminophylline in amounts in which the theophylline content was the same as above (114 mg/kg, ip) for 10 min, resulted in a significant increase in the percentage of phosphorylase *a* activity, 36.9 ± 1.9 ($n = 8$) compared with 26.0 ± 1.5 ($n = 4$) in the controls. It is likely that the effect of theophylline to elevate uterine phosphorylase *a* activity *in vivo* is only transitory which in effect is similar to that reported for epinephrine injections (5).

Discussion. Studies on the interaction and effects of theophylline, catecholamines and C-AMP on uterine smooth muscle have been primarily concerned with inhibition of uterine motility (1, 2). Metabolic changes induced by these agents have been investigated predominantly in liver, and in cardiac and skeletal muscles and the effect on phosphorylase activity has been one of these metabolic parameters (8, 9). It is generally accepted that catecholamines activate adenyl cyclase to produce C-AMP which in turn mediates the specific physiological and metabolic responses. Theophylline, by inhibiting a phosphodiesterase, permits an accumulation of C-AMP in receptive tissues to produce an effect similar to that obtained with catecholamines (8, 9). Uterine smooth muscle is unique as a target tissue in that it is subject to modification with sex hormones which conceivably could modify the metabolic effect of these agents.

A greater net increase in phosphorylase *a* activity after theophylline treatment occurs in estrogen-dominated uteri than in those which are not and this may be correlated with differences in uterine catecholamine levels. Epinephrine is increased in rat uteri after estrogen treatment and is higher during estrus than in diestrus (10). Epinephrine in circulation as well as its uptake by the rat uterus is greater during estrus (11, 12). It might be expected that C-AMP likewise would be elevated in the uterus after estrogen stimulation and would remain so up to the time of exposure to theophylline. An estrogen injection produces only a transient elevation of C-AMP (13) and of adenyl cyclase activity which is over within 2 hr (14). However, catecholamines were implicated in this transitory rise in C-AMP (14). Estrogen-primed uteri exposed to 10 m*M* theophylline for 20 min increased C-AMP above a low base level and more than exposure to 5×10^{-7} *M* epinephrine, but given in combination, a synergistic response increased C-AMP by 300% (15). No reference was made to similar studies in ovariectomized rat uteri. In any event, it would not seem logical for estrogen to maintain a high level of active phosphorylase at a time the hormone is stimulating glycogen deposition (16). Once uterine phosphorylase *a* activity is increased, the effect of estrogen is to prolong the time it takes for the active enzyme to revert to the inactive form (17). This occurs during the assay despite the addition of ethylenediaminetetraacetic acid to all homogenates which also inhibits the loss of enzyme activity during the assay procedure (17). Since estrogen treatment increases the binding capacity of the rat uterus for epinephrine (18), it is

possible that theophylline binding may also be increased which could enhance the effectiveness of this agent on residual C-AMP.

When the rats were subjected to reserpine treatment which is reported to reduce the catecholamine content in the uterus (6), the effect of theophylline on the enzyme activity was the same as in the controls. Since reserpine does not deplete the catecholamines completely from the uterus (6), the difference in the amounts of catecholamines remaining in experimental and control uteri may not have been great enough for theophylline to produce a differential response. The uterus may resemble the heart in so far as giving anomalous responses to reserpine in the activation of phosphorylase (9).

An increase in C-AMP in the uterus induced initially by epinephrine and the prevention of its disappearance by theophylline can explain the greater net increase in phosphorylase *a* activity over that produced by theophylline alone. Since oxytocin does not increase C-AMP in the uterus (19), theophylline was unable to maintain a level of enzyme activity greater than in the control. DB-C-AMP alone increased phosphorylase *a* activity by 35% in 3 of 4 estrogen-primed uteri but was unable to do so in uteri from ovariectomized rats. A difference in the ability of transport of DB-C-AMP into the cells could explain these results.

The mechanism by which theophylline is reported to inhibit uterine motility (19) seems applicable, in general, to explain the effect of theophylline on uterine phosphorylase. Of the two test systems, inhibition of uterine motility appears to be more sensitive. However, the mechanism of action of theophylline as presented has been questioned because theophylline potentiates the inhibitory effect of nitroglycerine as well as norepinephrine on uterine motility and pretreatment with nitroglycerine also potentiates the response to norepinephrine (2). These investigators suggest theophylline acts as a nonspecific potentiator of the effect of norepinephrine. Since nitroglycerine is unable to increase phosphorylase *a* activity in the uterus (20, and confirmed, unpublished) a further study on the interaction of nitroglycerine with theophylline and catecholamines on activiation of the enzyme is in order.

Summary. Theophylline at 5×10^{-3} *M* concentration acting for 20 min produced a greater net increase in phosphorylase *a* activity in isolated estrogen-primed uteri than in uteri from ovariectomized rats. This difference cannot be explained only by the reported elevated amounts of epinephrine in uteri stimulated by estrogen. Theophylline potentiated the effect of epinephrine treatment *in vitro* to increase uterine phosphorylase *a* activity but did not potentiate the effect of oxytocin. Pretreatment with reserpine which is reported to lower endogenous catecholamine levels in the uterus, did not decrease the effect of theophylline on the activity of the enzyme. Cyclic-AMP which mediates the effect of catecholamines to inhibit uterine motility and to increase phosphorylase *a* activity in other tissues, increased the activity of the enzyme only in the estrogen-primed uteri. Theophylline is reported to produce its effect by permitting an accumulation of C-AMP in responsive tissues.

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