Deciduogenic effects of mediators of the polyphosphatidylinositol pathway in pseudopregnant mice

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Abstract. Intraluminal injections (15 µl) of either concanavalin A (125 µg) or ionophore A 23187 (0.01 µmol) induced a decidual cell reaction (DCR) in the uterus of day 4.5 pseudopregnant mice. However, when these agents were administered in different combinations with each other or with $CaCl_2$ (15 µmol) and phorbol-12-myristate-13-acetate (1.6 nmol), interacting effects occurred to either enhance or inhibit each of the others' independent deciduogenic capacities. The results suggest that the polyphosphatidylinositol pathway and Ca^{2+} are involved in the induction of the DCR in mice with complex interactions occurring between the active components of the pathway to modulate the outcome of the transformation process.
Key words. Decidualization; pseudopregnancy; uterine biochemistry; polyphosphatidylinositol pathway; concanavalin A; calcium ionophore; phorbol ester.

The uterine decidual cell reaction (DCR) in many animals involves cellular changes in the uterus in response to either the presence of an implanting blastocyst or various artificial deciduogenic stimuli and is a necessary process for the establishment of pregnancy[1]. The nature of both the embryonic signal and the metabolic pathways and processes subsequently recruited in the uterine tissues to ultimately effect implantation and decidualization remains the subject of considerable speculation. The uterine luminal epithelium is considered to be an obligatory transmitter of the deciduogenic stimulus[2] to the underlying stromal cells and it is likely, therefore, that intracellular signals involving membrane-receptor-mediated events are involved in the process. Among the various components of intracellular signal transduction pathways that have been implicated in uterine events during the peri-implantation stages of pregnancy, cAMP and prostaglandins together with their associated enzymes adenylate cyclase and phospholipase A_2(PLA_2) have received the most attention[3]. Although some consideration has also been given to the role of the polyphosphatidylinositol pathway in this context[4], insufficient information is yet available to unequivocally implicate this process in uterine processes leading to ovo-implantation and the establishment of pregnancy.

Various receptor-mediated stimuli are known to cause the hydrolysis of inositol phospholipids[5] via the activation of membrane phospholipases resulting in the release of second messengers in the form of 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). Since IP_3 and DG elicit physiological responses by promoting the release of Ca^{2+} from intracellular stores and activating protein kinase C (PKC), respectively, it is apparent that a relationship exists between the activity of these pathways and cellular Ca^{2+}. An important role for Ca^{2+} in the embryonic signal responsible for the induction of the DCR in mice has been implicated[6] by the deciduogenic effect of compounds, such as lectins, which can induce polyphosphatidylinositol hydrolysis[7], and calcium ionophore A 23187, which in common with the plant lectin concanavalin A (Con A), promotes both the intracellular release of Ca^{2+} from intracellular stores and the ionophoretic transfer of Ca^{2+} across the plasma membrane[8,9]. Calcium ions, together with calmodulin, the intracellular receptor protein for calcium, play important roles as cell regulators[10], and the activation of PKC by DG has several functions, such as, phosphorylation of a number of cytosolic proteins, including lipocortin which is involved in activation of PLA_2[8,11]. PLA_2 hydrolyses phospholipids producing arachidonic acid (AA) required for the formation of prostaglandins. However, activation of this enzyme is not the only means of producing AA since hydrolysis of polyphosphatidylinositol by phospholipase C and the resulting DG by diglyceride lipase also release AA[12,13]. Thus, while it is apparent that relationships exist between the polyphosphatidylinositol pathway and alterations of Ca^{2+}, prostaglandins and cAMP in a variety of cell types[14–17], similar associations have yet to be demonstrated in the uterus during early pregnancy. However, since the production of prostaglandins[2,18], particularly prostaglandin E-2 (PGE-2)[19] and the concentration of PGE-2 receptor sites[20] appear to be important in the DCR by involving the adenylate cyclase system and cAMP[21,22], the potential involvement of the polyphosphatidylinositol pathway in this capacity is highly feasible.

The effects of the second messengers can be mimicked by phorbol ester activation of PKC and ionophore A 23187 release of intracellular Ca^{2+}[5,11,23]. In addition, Con A mediates the hydrolysis of polyphosphatidylinositol and promotes both the uptake and intracellular release of Ca^{2+}[24,25]. Earlier results of work by Buxton and Murdoch[6] showing that plant lectins and A 23187 can initiate a DCR in pseudopregnant mice, suggest a role for this signal transduction pathway in the uterine transformations accompanying blastocyst implantation.

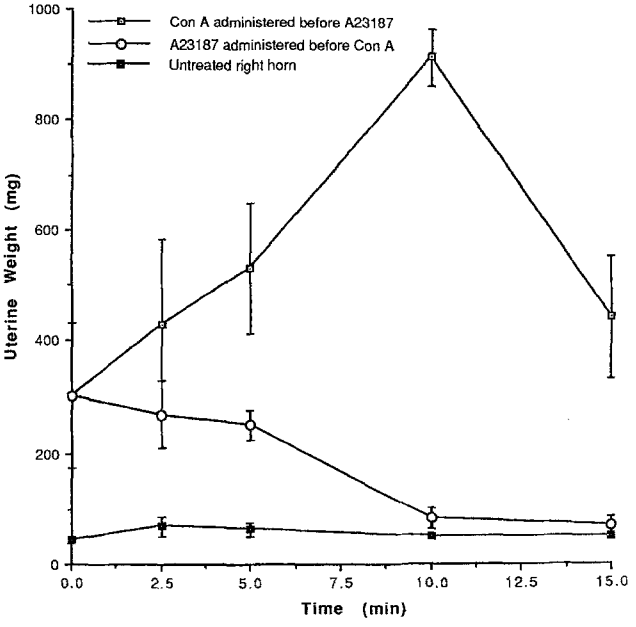
In view of these considerations, the present study was designed to test this hypothesis by examining the deciduogenic actions of mediators of the polyphosphatidyli-

nositol signal transduction pathway in pseudopregnant mice.

Materials and methods

Female Quackenbush strain mice, aged 6–10 weeks and maintained in a controlled environment, were used in all experiments. Pseudopregnancy was brought about by pairing females with vasectomised males. The females were examined for copulation plugs each morning, and the day of finding a plug was designated as day 1 or the first day of pseudopregnancy.

The ability of phorbol ester, ionophore A 23187, Con A and $CaCl_2$ to induce uterine deciduomata was examined, unless otherwise indicated, by injecting 15 µl of each agent in solution into the uterotubal junction of the left uterine horn of mice between 12.00 and 15.00 h on day 4 of pseudopregnancy after anaesthesia with Avertin. The ovaries of all pseudopregnant mice were examined under a dissecting microscope before the intrauterine injections were administered, and animals were used in experiments only when distinct corpora lutea were apparent. The right uterine horn in all animals received no treatment and acted as a control in the experiments. When two treatments were separated by time, the incision was covered with cotton wool soaked in 0.9%(w/v) NaCl while the body temperature was maintained by placing the animal on a warming tray at 37 °C. Investigations of the effects of separating treatments by time were conducted because previous studies demonstrated that priming or prior exposure of various cell types to either Con A, phorbol ester or A 23187 profoundly influenced the response to later stimulation with the above agents [24, 26]. In most cases, a 10-min period was selected to separate treatments spaced by time since preliminary tests revealed that, if interacting effects of the agents were exerted on the uterus, they were maximal at this time. This is supported by the results presented in the figure. Consequently, the results presented in the table documenting effects of agents separated by time relate only to a 10-min time period. The mice were killed by cervical dislocation on day 9 when the right (untreated) and left (treated) horns were excised, dissected free of all connective tissue and individually weighed. All experiments were conducted using a minimum of 4 mice in each treatment group.



Changes in uterine weight with increasing interval between treatments.

The significance of results was assessed by paired comparison t-test after converting the primary data to logarithms. The multiple range test (Duncan, 1955) was also used for comparisons among means. All values listed are means ± standard error of the mean unconverted data. Phorbol-12-myristate-13-acetate (PMA, Sigma Chemical Co., St Louis, USA) and calcium ionophore A 23187 (Calbiochem-Behring, Australia) were dissolved in dimethyl sulfoxide (DMSO, AJAX Chemicals, Australia) and diluted in 0.9% NaCl to permit 1.6 nmol and

Uterine weight of the left horn of day 9 pseudopregnant mice after intraluminal injection of agents on day 4.5. Values represent the means ± SEM

Treatment A	Treatment B	Uterine weight (mg) with: A alone	A + B together	A injected 10 min before B	B injected 10 min before A
DMSO	–	55 ± 4	–	–	–
Peanut oil	–	605 ± 70**	–	–	–
NaCl	NaCl	42 ± 5	40 ± 4	–	–
CaCl	$CaCl_2$	34 ± 5	48 ± 8	67 ± 16	–
A 23187	A 23187	333 ± 44**	300 ± 45**	308 ± 65**	–
PMA	PMA	100 ± 15**	90 ± 10*	83 ± 10	–
Con A	Con A	361 ± 92**	350 ± 70**	328 ± 72**	–
NaCl	Con A	–	360 ± 90**	274 ± 33**	502 ± 51**
$CaCl_2$	Con A	–	50 ± 4[a]	189 ± 38***	951 ± 51[a]***
A 23187	Con A	–	302 ± 128[b]**	83 ± 20[b]	913 ± 51[b]**
A 23187	PMA	–	179 ± 23*	135 ± 32*	299 ± 59**
PMA	Con A	–	152 ± 53[c]*	424 ± 158[cd]*	142 ± 36[d]

Combined values of all untreated right horns = 45 ± 5

*, ** Significantly different from the right untreated horn, $p < 0.05$, $p < 0.01$, respectively; [a, b, c, d] represent significant differences between compared treatments; $p < 0.05$.

0.01 µmol, respectively, to be injected into the uterine horns as described above. Con A (Pharmacia South Seas Pty Ltd, Australia) was dissolved in 0.9% NaCl to permit the injection of 125 µg of the lectin, while 15 µmol $CaCl_2$ was administered from a 1 M $CaCl_2$ solution. These concentrations were utilised because they had been established either in preliminary trials with PMA and $CaCl_2$ (data not shown), or in previously published reports in the case of A 23187 and Con A[6]. Solutions of 0.9% NaCl either containing or not containing DMSO in amounts comparable to those of the PMA and ionophore solutions were similarly injected into the uterine horns of pseudopregnant control mice to test for any inherent deciduogenic activity of the carrier solutions.
Peanut oil is a potent stimulus of the decidual cell reaction in pseudopregnant mice[27] and, because its use in this respect is so widely documented, it was used in the present study to standardize and guage the effectiveness of other treatments. The oil was administered as described above using a 30-µl injection into the left uterine horn.

Results

The effects of mediators of the polyphosphatidylinositol signal transduction pathway, peanut oil and carrier solutions containing DMSO on the decidual cell reaction in the mouse uterus are shown in the table. DMSO, NaCl and $CaCl_2$ did not show evidence of deciduoma formation since the weights of treated (left) and untreated (right) uterine horns did not differ signficantly. Peanut oil, however, did induce a decidual response characterised by a large, significant ($p < 0.01$) increase in uterine horn weight. The morphological appearance and weight of the right untreated uterine horns at all times remained unchanged irrespective of the treatment administered to the left uterine horn, indicating that the deciduogenic effects of the agents were confined to the treated horns. When either calcium ionophore A 23187, which increases intracellular Ca^{2+} concentration[8,9], PMA, as an activator of PKC[5], or Con A, which induces the hydrolysis of polyphosphatidylinositol[7], were individually introduced into the left uterine horn of pseudopregnant mice, significant ($p < 0.01$) increases in uterine weight occurred, but the magnitude of the response to PMA was not as great as that induced by peanut oil, A 23187 or Con A.
The table also shows the effects of combinations of the agents which were administered to investigate possible interactions that may implicate both the IP_3 and PKC arms of the polyphosphatidylinositol pathway[11,28] in the DCR. In this case, two administrations each of NaCl, $CaCl_2$, A 23187, PMA or Con A, given either simultaneously (A + B together) or spaced 10 min apart (A injected 10 min before B) produced uterine responses which failed to differ significantly from those induced by the agents administered as single doses (A alone). Since these results clearly indicated that the mechanics involved in the intrauterine administration of two agents, whether given simultaneously or separated in time, would not be a confounding factor in this procedure, the effects of mixed paired combinations of the compounds in the DCR were examined. All combinations of saline (0.9% NaCl) and Con A produced significant ($p < 0.01$) increases in uterine weight, but the magnitude of the responses did not differ significantly from that induced by Con A alone. In contrast, combined treatments of either $CaCl_2$ and Con A, or A 23187 and Con A induced uterine responses which differed in magnitude depending on the order of uterine exposure to the agents. Thus, when Con A was administered first, followed 10 min later by either $CaCl_2$ or A 23187, the increase in uterine weight was significantly ($p < 0.05$) greater than that produced by reciprocal or simultaneous combinations. However, unlike the total inhibition of the DCR achieved either with A 23187 administered 10 min before Con A, or with $CaCl_2$ administered together, $CaCl_2$ injected 10 min before Con A induced a significant ($p < 0.01$) increase in the weight of the treated left uterine horn in comparison with the untreated right uterine horn.
In order to further investigate the combined effects of Con A and A 23187, intraluminal administrations of Con A and A 23187 were separated by 2.5 min, 5 min, 10 min and 15 min as shown in the figure. Con A-treated uteri which were subsequently exposed to A 23187 showed significant increases in weight as the time between the treatments increased, with the maximum response occurring at 10 min. A reversal of this effect was observed when A 23187 was injected before Con A. In this case there was a total inhibition of the decidual response when the lectin was administered either 10 or 15 min after the ionophore with the untreated right and treated left horns showing no significant difference in weight.
The data presented in the table also show that reciprocal administrations separated by 10 min of PMA and A 23187 were not significantly different from each other or from the simultaneous uterine administration of the compounds. However, all treatments with PMA and A 23187 induced significant ($p < 0.05$) increases in uterine weight. Furthermore, when PMA was administered 10 min before A 23187, the uterine response failed to differ significantly from that induced with peanut oil and was greater ($p < 0.05$) than that induced by PMA alone. In addition, reciprocal treatments separated by 10 min of PMA and Con A also produced significant effects ($p < 0.05$) on uterine weight. Thus, an initial intrauterine administration of PMA 10 min before Con A induced an increase ($p < 0.05$) in uterine weight which was comparable to that of peanut oil or Con A administered individually but greater than the reverse treatment of PMA and Con A. The response induced by treatment with Con A 10 min before PMA was not significantly different from that induced either by the simultaneous administration

of Con A and PMA or by PMA alone but was significantly lower ($p < 0.05$) than that induced by Con A alone.

Intrauterine administrations of the above compounds on either days 3 or 5 of pseudopregnancy, when the uterus is not hormonally-sensitized for decidualization [29], failed to induce any significant increases in uterine weight or morphological changes (data not presented).

Discussion

The results of the present study clearly demonstrate that compounds, such as Con A, A 23187 and PMA, that either stimulate the activity of the intracellular polyphosphatidylinositol signal transduction pathway or mimic the action of its second messenger components, are capable of inducing a DCR in the uterus of hormonally-sensitized pseudopregnant mice, with the magnitude of the response varying with different combinations of the compounds. The failure of any of the compounds, either singularly or in combination, to induce significant changes in uterine weight or morphology in day 3 and day 5 pseudopregnant mice, which were not hormonally-sensitized for induction of the DCR, indicates that the effects are confined only to the period on day 4 post coitum when the uterus is transiently prepared by hormones to register and respond to the deciduogenic signal from the blastocyst [29].

The deciduogenic properties of Con A observed in the present investigation were also reported in a previous study [6] in which it was shown that the intraluminal administration of the lectin, either in free form or covalently bound to sepharose 4B beads, stimulated the formation of deciduomata in hormonally-sensitized uteri. On binding to the plasma membrane glycoprotein residues, Con A influences cellular functions by stimulating the rate of hydrolysis of phosphatidylinositol-4,5-bisphosphate ($PtdInsP_2$) [7]. The hydrolysis of $PtdInsP_2$ produces two second messenger molecules, IP_3 and DG which, in turn, influence cellular behavior through activation of protein kinases [8], as described in the introduction. It has been reported that the Con A-induced turnover of $PtdInsP_2$ leads to the phosphorylation of the same cytosolic proteins in rat mast cells as does the phorbol ester-activation of PKC [30]. Consequently, the deciduogenic effects of Con A in the pseudopregnant mouse may be explained by these mechanisms.

Cells generally require the activation of both the DG-PKC and IP_3-Ca^{2+} divisions of the polyphosphatidylinositol pathway to elicit a maximal response [5,11], and it has been established that similar synergism can be achieved artificially by the phorbol ester activation of PKC and increase of intracellular Ca^{2+} by calcium ionophores [26, 31, 32]. The results of the present study suggest that activation of both divisions of the pathway may also be required in the pseudopregnant mouse uterus to facilitate full decidualization since the responses induced by treatment with either PMA, a phorbol ester that mimics DG in its capacity to activate PKC [33], or A 23187, a calcium ionophore that mimics the action of IP_3, were submaximal when compared with those induced by peanut oil or combinations of compounds involving Con A. However, the nature of this relationship appears complex. Thus, while the results suggest that the activation of PKC alone is not sufficient for full induction of the DCR in mice, the activation of the uterine enzyme by PMA nevertheless modifies the magnitude of the DCR, at least in response to Con A and A 23187.

The varying deciduogenic effects of the different combinations of $CaCl_2$, Con A, A 23187 and PMA further indicate that the relative timing of the PKC activation and intracellular Ca^{2+} release is critical in second messenger signalling of the DCR in mice. In support of this proposal, Berridge [34] reviewed evidence to suggest that PKC activation and Ca^{2+} mobilization may not be achieved simultaneously and that the concentration of intracellular Ca^{2+} may vary as a series of oscillations. It has also been suggested that the generation of IP_3 is not immediate in some stimulated cells [35] and that the behavior of PKC is complicated by the membrane binding properties of the enzyme [36]. Although the intracellular release of Ca^{2+} through IP_3 effects, as mimicked by A 23187, appears to be sufficient to induce a DCR in pseudopregnant mice, it should be stressed that the response was either completely inhibited or enhanced in the present study by additional stimuli critically timed in relation to the A 23187 stimulus. Consequently, while it appears that Ca^{2+}-mediated events may be major mechanisms governing the induction of the DCR, the present results suggest that they can be modulated by stimulation of PKC such as to influence the magnitude of the cellular response. On extrapolating these phenomena to the state of early pregnancy, it is tempting to suggest that the uterine luminal epithelium may be similarly stimulated at implantation sites to facilitate the relay of the deciduogenic blastocyst signal to the underlying stroma, and then become refractory to further stimulation in order to prevent the implantation of other blastocysts at the sites.

In keeping with the present results, Taylor [24] found that the preincubation of thymocytes with Con A inhibited Con A-induced IP_3 production and suggested that Ca^{2+} was involved in regulating the metabolism of the phosphoinositides. Since increases in intracellular Ca^{2+} can also lead to alterations in the production of cyclic AMP and prostaglandins [37], compounds which are now considered to play vital roles in decidualization [18, 22], metabolic links between these agents and the polyphosphatidylinositol pathway [14–17] may form at least part of the mechanistic complex through which the blastocyst signals the establishment of pregnancy and the induction of the DCR.

The present results conflict with the claim by Feyles and Kennedy [4] that the intrauterine infusion of A 23187 in rats inhibits the DCR. This discrepancy may be due to species differences, but since those authors acknowledged

that the infusion vehicle itself was deciduogenic in rats, antagonistic effects between the deciduogenic stimuli, similar to those described in the present study, provide the most likely explanation for the inhibitory action of the ionophore in this case.

In conclusion, the present results strongly suggest that the polyphosphatidylinositol pathway in the hormonally-sensitized uterus of mice plays an important role in the transduction of deciduogenic signals from the lumen to the stroma to effect the DCR and the establishment of pregnancy. However, it appears that complex and sensitive arrangements between the second messenger molecules involve the pathway in this capacity, and further work is required to elucidate the precise mechanism of the process.

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Effect of monensin and diabetes on asialoglycoprotein degradation in rat hepatocytes

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Abstract. We have studied the effects of two modulations – streptozotocin-induced diabetes in vivo, and the presence of the carboxylic proton ionophore monensin in vitro – on the degradation of ^{3}H-asialoorosomucoid ligand in isolated rat hepatocytes.

The ligand was internalized by means of a synchronous wave procedure. Diabetes was associated with a marked decrease in the amount of total degraded radioactive ligand compared to that in normal cells (3.6% and 37.3% of internalized ligand respectively, at 60 min), together with increased secretion of degradation products into the incubation medium (87% and 46.3% of the total degraded ligand was secreted by diabetic and normal cells, respectively). Monensin induced similar effects in normal cells, but had no apparent effect in diabetic cells.
Key words. Hepatocytes; asialoglycoprotein receptor; degradation; diabetes; monensin.