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INFLUENCE OF HORMONES ON PLATELET INTRACELLULAR CALCIUM

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Abstract The pathophysiology of thromboembolic disease associated with estrogen therapy is poorly understood. There are innumerable calcium-dependent activities involved in platelet function. To determine whether platelet calcium levels are affected by exogenous hormones, intracellular calcium and release were studied in platelets in various hormonal environments and findings were correlated with platelet adhesion and aggregation. Platelet intracellular calcium concentration and release was significantly decreased in women ingesting tamoxifen compared to controls and significantly increased, as was platelet adhesion, in oral contraceptive users. Platelets incubated ex vivo with estradiol had increased intracellular calcium and release but there was decreased adhesion to fibronectin. Intracellular calcium concentration and release were not affected when platelets were incubated with tamoxifen. Adhesion to collagen III was increased in tamoxifen-incubated platelets. Only oral contraceptive users had increased sensitivity to aggregating agents. This data suggests that 17ß estradiol, progesterone, and tamoxifen likely have a nongenomic effect on platelet intracellular calcium and calcium release and that platelet calcium levels are closely related to the degree of platelet adhesion and aggregation in vivo.

Key words: platelet intracellular calcium; calcium release; 17ß estradiol; tamoxifen citrate; oral contraceptives; platelet adhesion.
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Numerous reports have clearly linked estrogen therapy with "hypercoagulability". Included among these are the use of oral contraceptives (OC) containing more than 100 μg of estrogen (1,2), the use of high dose estrogen in the treatment of prostate cancer (3,4), and the use of estrogen to prevent coronary heart disease in men (4). Controversy remains as to whether or not tamoxifen citrate (TAM), an antiestrogen with partial estrogenic effects, causes or contributes to thromboembolic disease (5-7). We have previously reported that women who consume OC have markedly increased platelet adhesion compared to normal individuals (8). Morley et al have reported that estrogen causes a rapid release of intracellular calcium ($Ca^{2+}_{(i)}$) from chicken granulosa cells (9). They postulate that the mechanism of this rapid $Ca^{2+}_{(i)}$ release is due to a nongenomic action triggered by a signal generating receptor on the cell surface rather than by a gene-activating nuclear steroid receptor complexes. The ensuing studies were done to determine the relationship between platelet $Ca^{2+(i)}$ levels and platelet adhesion/aggregation using both an ex vivo and in an vitro model. We measured platelet $Ca^{2+}_{(i)}$ in women ingesting either OC or TAM and compared these levels to those from platelets of normal donors. Platelet $Ca^{2+}_{(i)}$ and calcium release was also measured after in vitro incubation of platelets with 17ß estradiol (ESTR), progesterone or TAM. The relationship between platelet $Ca^{2+}_{(i)}$ levels, Ca^{2+} release, aggregation and adhesion to fibronectin or collagen III was studied in each group.

MATERIALS AND METHODS

The study was approved by the Human Subjects Review Committee of Memorial Hospital of Rhode Island. Normal men and premenopausal women, women on OC and women with early stage breast cancer ingesting TAM as adjuvant drug therapy (10 mg twice a day) were studied. No medications known to affect platelet function or calcium channel blockers were consumed by these individuals.

Platelet Preparation.
Venous blood was collected into 1/10 volume of 3.8% sodium citrate from subjects who had not ingested aspirin for at least 10 days prior to study. Platelet rich plasma (PRP) was prepared by centrifuging the whole blood at 1000g x 10 minutes with subsequent removal of the PRP. All platelet studies were performed at room temperature. Platelet counts were adjusted to 300,000/μl using platelet poor plasma (PPP). Platelet aggregation studies were performed using standard methodology as previously described (10).

Platelet Incubation Studies.
Platelets from normal subjects were incubated in TAM (1.0 μg/ml), kindly supplied in pure form by ICI Pharmaceuticals, or methanol (METH), the diluent in which the TAM was solubilized. To insure that the TAM was in solution, a small volume was sonicated before it was added to the PRP. Platelets were also incubated in ESTR (Sigma), 1 ng/ml or progesterone (Sigma) 10^{-5}M. 17ß estradiol and progesterone were each diluted in PBS. All incubations were performed at 37°C for 30 minutes prior to each flow study.

Measurement of $Ca^{2+}_{(i)}$.
Citrated whole blood was centrifuged at 1000 RPM for 10 min. 10% ACD was added to the PRP and the sample was respun at 2800 RPM for 10 min. The sample was washed with PBS/10% ACD and resuspended in PBS with 1% glucose at a concentration of 10^9 platelets/ml. A sample of platelets was removed for the control preparation and 1 mM fura-2AM (Sigma) (dissolved in 1 mM DMSO) was added to the sample and incubated at 37°C for 1 hr. The free fura-2AM was removed by washing with PBS/10% ACD and the PRP was resuspended in PBS with 1% glucose at a concentration of 50,000 platelets per µl. The PRP was then incubated with ESTR, METH, progesterone or TAM for 30 minutes, washed and resuspended in PBS with 1% glucose. For calcium channel blocking studies, a 10 minute incubation with verapamil (50 µM) or neomycin (1.5 mM) was performed prior to incubation with ESTR. The sample was placed in a fluorescence spectrophotometer (37°C) and readings obtained at 510 nM with excitation at 340 nM and 380 nM. Triton X-100 (0.1%) and ethyleneglyco-bis-tetracetic acid 100X (EGTA) were added to the samples. Thrombin (1 U/ml) was added to each sample and readings obtained at both wavelengths over 5 minutes. Maximum fluorescence was obtained after the addition of Triton X-100 and minimum fluorescence after the addition of EGTA. The ratio (R) of the measured fluorescence values at 340 nM and 380 nM excitation was calculated. Cytosolic free calcium concentration was calculated by the method of Grynkiewicz et al (11) and Tepel et al (12): $Ca^{2+}_{(i)} = K^* (R\text{-}Rmin)/(Rmax\text{-}R)$, where Rmin is the ratio of calcium free solution, Rmax is the ratio at saturation and K is 113 nM/l F min 2/F max 2. The latter is the fluorescence minimum and maximum at 380 nM excitation.

Flow Studies.
Platelet adhesion studies were performed in a Hele-Shaw flow chamber between two, flat, parallel surfaces as previously described (13). The chamber itself is a machined piece of polymethylmethacrylate (plexiglass) and is 43 cm in length, 14.2 cm in width and has a 0.4 mm gap between the two surfaces. The lower, flat surface of the channel is formed by the deck of the chamber, and a glass slide (75 x 38 mm) constitutes the upper surface. The slide is held in place by suction from a perimeter vacuum pump. Inflow and outflow ports for the PRP sample are located at either end of the chamber. The PRP sample is maintained at 37°C by a thermistor in an additional port opposite the inlet port and attached to a telethermometer. The chamber is mounted on the stage of an inverted microscope with a Hoffman Modulation Contrast System (40x objective, 0.55 numerical aperature) which provides a 3-dimensional image of cells and thus facilitates platelet identification. The flow is regulated and the PRP sample is pumped through the chamber by a syringe infusion pump (model 940, Harvard Apparatus Co., Inc., Millis, MA). Shear rates were calculated according to Batchelor (14). To record the adhesion process, a Nikon 35mm camera is mounted on the microscope and focused on the inside glass surface of the coated slide. Photographs of the experimental run were taken at 30 second intervals for 12 minutes. A shutter speed of 1/4 second allows stationary platelets attached to the slide to remain in focus while moving platelets are blurred. Platelets are observed to attach and also to detach. Once a site has been occupied by a platelet it is more likely to be reutilized as an adherent site by other platelets. Platelet spreading (platelets adjacent to each other) was noted, but rarely was there aggregate formation. All platelets could be individually enumerated.

Computer-aided analysis was performed on developed photomicrographs from each experiment. Black and white negatives (Tri-X Pan 400) were projected with a 35mm film projector onto a Summagraphics microgrid digitizing table. The location of each platelet was identified with a cursor and each platelet was registered with distinct coordinates. Using a Digital Equipment Corporation VT 100 terminal connected to a VAX II computer system and a modified computer program written in BASIC by Kane (15), the following information was provided at each time point; 1) occupied sites, i.e. the total number of sites occupied by platelets at each time point; 2) cumulative sites, i.e. a running total of fresh adhesions; and 3) total number of platelets adherent to the surface at 10 minutes.

Coating Slides with Collagen III and Fibronectin.
Human fibronectin (Sigma) and collagen III (Chemicon) were purchased in lyophilized form. Fibronectin was reconstituted with distilled H_2O (0.5 mg/ml) and collagen was reconstituted with acetic acid (0.1 M). Glass slides (75 x 38 mm) were cleaned by soaking for 5 hours in consecutive order: detergent, 1N KOH and 1N HCl. The slides were rinsed thoroughly with distilled water between each chemical and then air-dried. The slides were subsequently coated individually with 50 µl of fibronectin or collagen (spread evenly with a rubber policeman), allowed to dry, and stored at -20°C. Coated slides were left at room temperature for 30 min prior to each flow study. Platelet adhesion to collagen III was tested at several doses in order to select the optimum dose for adhesion. Ten µg/slide was the dose providing optimal adhesion for counting purposes. The data on fibronectin has previously been reported (8).

Statistical Analysis.
The data were analyzed using the following tests wherever indicated: t test; $\bar{x} \pm$ SEM $\pm$ SD; coefficient of variation; and analysis of variance.

RESULTS

Ex Vivo Studies.
Baseline $Ca^{2+}_{(i)}$ was significantly different ($p < 0.05$) between the groups studied [Fig. 1 and Table I (time = 0 min)]. Women ingesting OC had the highest baseline platelet $Ca^{2+}_{(i)}$ (126 nM/l) and the greatest release of calcium following maximal stimulation with thrombin ($p < 0.018$). Women ingesting TAM had the lowest baseline $Ca^{2+}_{(i)}$ (66.8 nM/l) of all groups tested ($p < 0.006$). Thrombin induced $Ca^{2+}_{(i)}$ release was not significantly different between men and women control groups. We have already reported that women ingesting OC have the greatest platelet adhesion at low shear rates of all normal groups tested (8) and women ingesting TAM have significantly lower platelet adhesion than do women taking OC (16). The platelet adhesion studies previously reported correlate with the $Ca^{2+}_{(i)}$ findings in the ex vivo platelet studies.

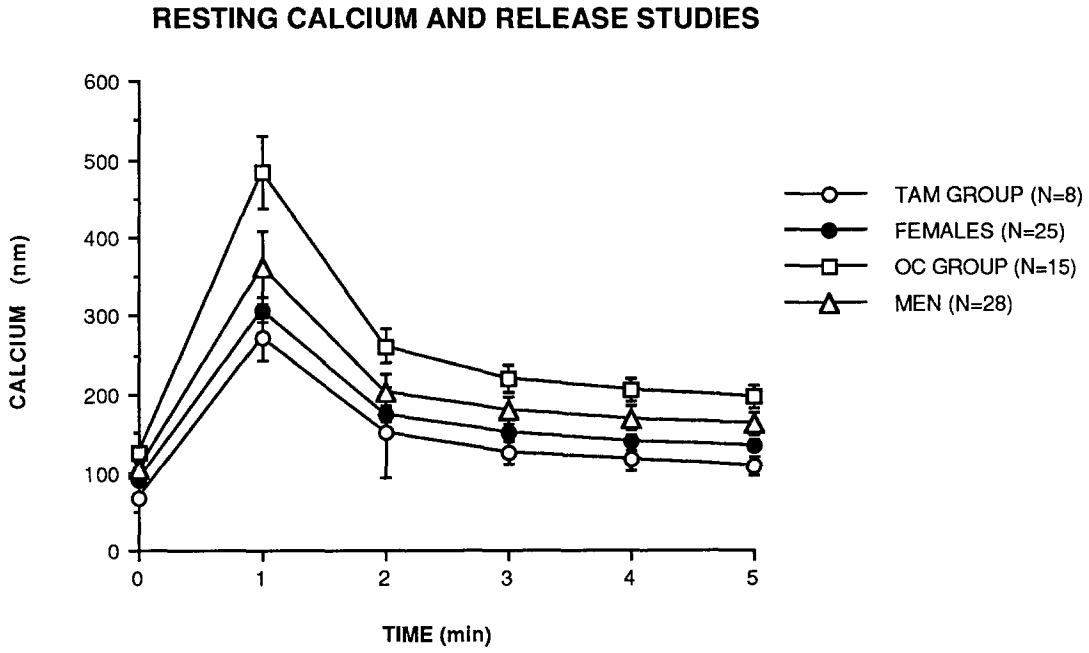

FIG. 1.

Resting $Ca^{2+}_{(i)}$ levels and calcium release in thrombin-stimulated platelets from normal controls, women ingesting tamoxifen or oral contraceptives. Data is expressed as $\bar{x} \pm$ SEM.

Table I

$Ca^{2+}_{(i)}$ (nM/l) in all groups compared to platelets incubated in 17ß estradiol (10 ng/ml).

GROUP	t(min)	CONTROL	ESTR
Female	0	90.3 ± 4.1 (25)*	184.6 ± 12.9 (13)*+
Male	0	105.3 ± 5.9 (28)	199.7 ± 10.3 (23)
OC	0	126.0 ± 6.3 (15)	231.5 ± 26.1 (7)
TAM	0	66.8 ± 5.7 (8)	----
Female	1	307.8 ± 15.9	517.8 ± 40.6
Male	1	362.2 ± 26.8	680.7 ± 84.4
OC	1	484.9 ± 45.8	828.6 ± 181.0
TAM	1	271.8 ± 27.5	----
Female	2	175.3 ± 9.4	339.1 ± 26.0
Male	2	204.1 ± 15.4	384.1 ± 31.2
OC	2	262.3 ± 21.6	580.7 ± 48.1
TAM	2	150.7 ± 20.5	----
Female	3	149.7 ± 7.7	301.7 ± 23.1
Male	3	180.0 ± 12.9	332.3 ± 25.4
OC	3	220.4 ± 16.9	485.0 ± 25.4
TAM	3	124.1 ± 14.9	----
Female	4	140.4 ± 7.6	229.0 ± 20.4
Male	4	168.7 ± 11.6	304.3 ± 21.7
OC	4	205.6 ± 15.4	419.1 ± 53.6
TAM	4	114.5 ± 13.1	----
Female	5	134.0 ± 7.1	262.1 ± 17.9
Male	5	161.7 ± 11.4	286.2 ± 19.9
OC	5	195.9 ± 14.3	380.9 ± 50.1
TAM	5	107.7 ± 11.3	----

* x ± SEM (n)
+ all incubations with ESTR significantly higher than control $p < 0.001$

In Vitro Incubation Studies.
Platelets from normal men, normal women and women taking OC were incubated in vitro with ESTR (10 ng/ml). In vitro ESTR incubation was associated with higher platelet resting $Ca^{2+}_{(i)}$ and greater release of $Ca^{2+}_{(i)}$ with thrombin stimulation compared to unincubated control platelets (Table I). The difference was statistically significant for each group of individuals studied and at every time point ($p < 0.001$).

Platelets from normal men were incubated with progesterone 10^{-5} M). Resting $Ca^{2+}_{(i)}$ was significantly increased ($p = 0.002$) in the progesterone-treated platelets compared to unincubated controls (Fig. 2). Calcium release at 1 and 5 minutes was not significantly different from control. Calcium release at all other time points measured was significantly higher in the platelets incubated in progesterone compared to controls ($p < 0.05$). Platelets from normal men were incubated with TAM (1 ng/ml). Resting platelet $Ca^{2+}_{(i)}$ and release did not differ from their control, unincubated platelets (Fig. 3). Platelets from normal men incubated with TAM showed increased platelet adhesion to collagen III (Table II). There was no difference in platelet adhesion to collagen III when platelets from normal men were incubated in ESTR, compared to unincubated controls. However, platelets from normal men incubated in TAM were more adherent to collagen III than unincubated controls ($p < 0.004$) (Table II). The $\bar{x} \pm$ SEM of the slope of the cumulative sites of control platelets adherent to fibronectin was significantly greater than the adhesion of platelets incubated in vitro with ESTR (Table III).

Table II

Adhesion of platelets from men to collagen III

Measurement	Control	ESTR (N = 6)	Control	TAM (N = 7)	p
Slope of cumulative sites	96 ± 22	115 ± 20*	65 ± 20	120 ± 16	0.004
Slope of occupied sites	13 ± 3	16 ± 3*	10 ± 3	16 ± 3	0.004
Adherent platelets at 10 minutes	115 ± 23	131 ± 22*	79 ± 22	136 ± 19	0.001

* = NS

Table III

Adhesion of platelets to fibronectin

	Slope of Cumulative Sites	P value
Control Platelets*	13.3 ± 2.7 (13)	< 0.02
Platelets and ESTR	7.9 ± 1.9 (13)	

* Source of platelets were normal men and premenopausal women.

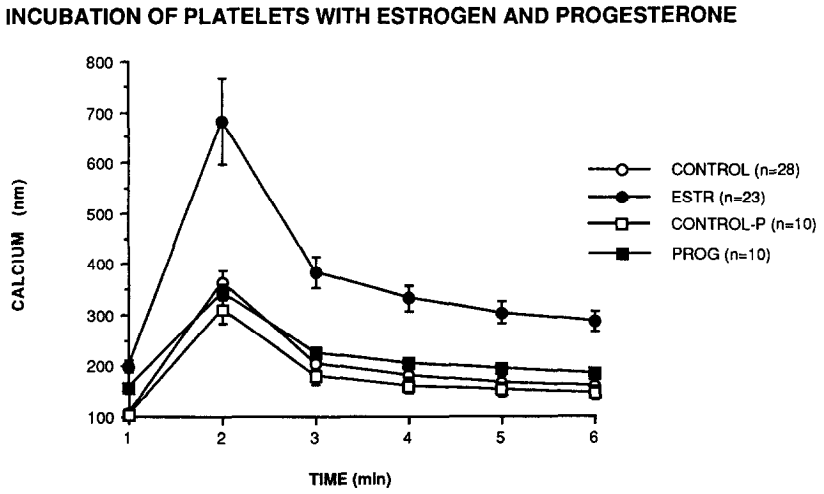

FIG. 2.

Platelets from normal individuals were incubated in vitro with 17ß estradiol or progesterone and resting $Ca^{2+}_{(i)}$ and release was measured. Open circles (control) represent the control group for the 17ß estradiol incubation. Open squares (control-p) represent the control group for the progesterone group. Data is expressed as $\bar{x} \pm$ SEM.

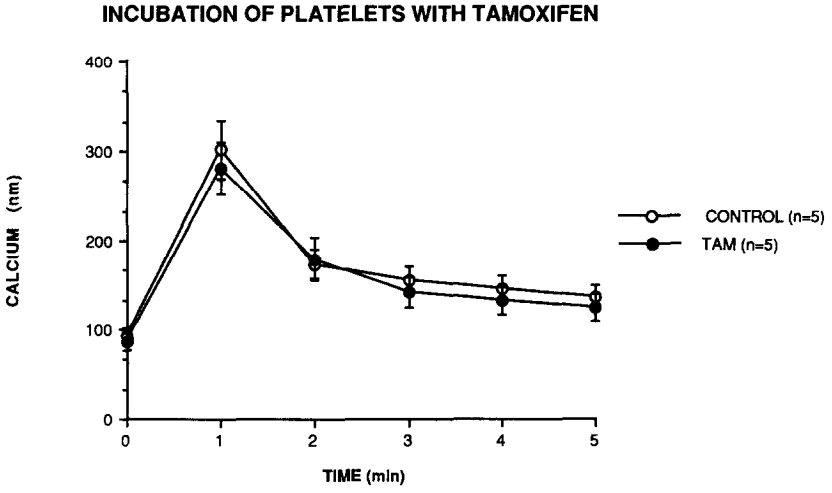

FIG. 3.

Resting $Ca^{2+}_{(i)}$ and calcium release in platelets from normal men incubated in vitro with tamoxifen. Data is expressed as $\bar{x} \pm$ SEM.

Ca^{2+} Channel Blocking Studies.
Incubating platelets from women ingesting OC with verapamil followed by addition of ESTR was not associated with an increase in resting platelet $Ca^{2+}_{(i)}$ or release (Fig. 4). The platelet $Ca^{2+}_{(i)}$ and release was comparable to controls, prior to the addition of verapamil. Similarly, when platelets from women taking OC were incubated with neomycin and then ESTR added, there was no effect on resting $Ca^{2+}_{(i)}$ and release with the addition of ESTR (Fig. 4).

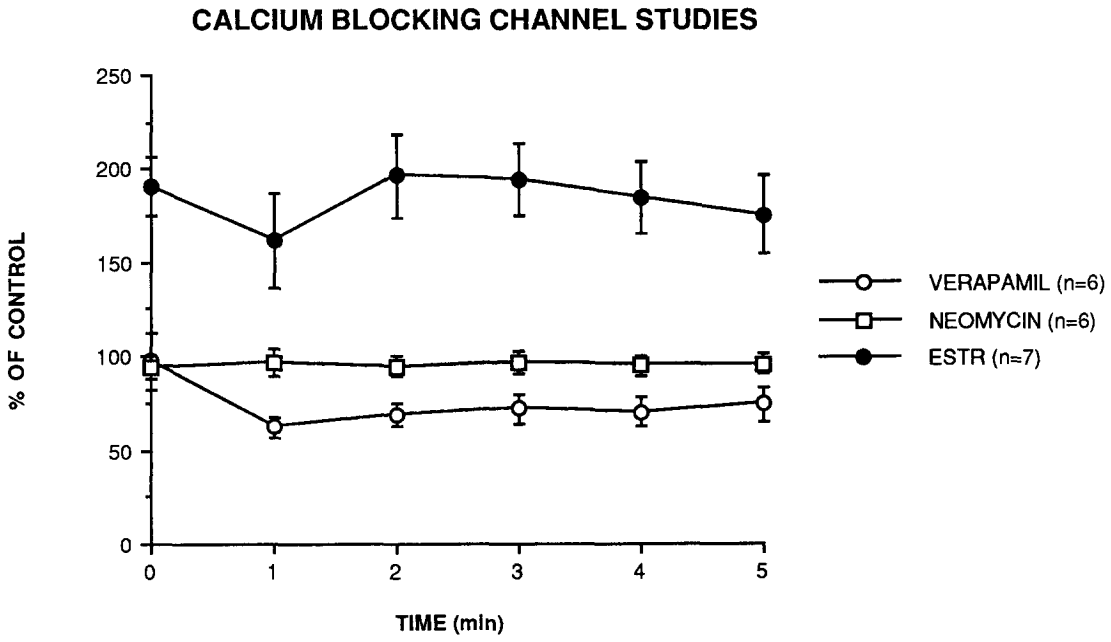

FIG. 4.

Resting $Ca^{2+}_{(i)}$ and release in platelets incubated in vitro with verapamil (open circles) or neomycin (open squares) followed by addition of 17ß estradiol. Platelets incubated in 17ß estradiol alone are represented by the closed circles. All groups were compared to control, unincubated platelets. Data is expressed as % control, $\bar{x} \pm$ SEM.

Aggregation Studies.
We have previously reported that women taking TAM have normal platelet aggregation and a normal platelet release reaction (16). Others have reported that women on OC have increased platelet aggregability (17-20). Our studies confirm the

latter. We found a significantly increased sensitivity of platelets from women on OC to collagen, ADP, epinephrine and arachidonic acid (data not shown). Incubation of normal platelets with ESTR or TAM did not change the aggregation sensitivity of the platelets to collagen, ADP, epinephrine or arachidonic acid.

DISCUSSION

For decades thromboembolic complications have been linked to estrogen use (1-4,21). Estrogen has multiple effects on coagulation factors and endothelial cells (21-23). Platelets and the subendothelial surface play an important role in thrombogenesis, both at high and low shear rates. Calcium is one of the major intracellular regulators of all cell processes, particularly in platelets where there are innumerable calcium dependent activities involved in platelet adhesion, spreading, shape changes, binding of fibrinogen, aggregation, secretion of the contents of storage granules and other processes. Platelet adhesion is the first step in thrombus formation (24). After platelets adhere to subendothelial matrix, they spread on the matrix (25). Fibronectin and collagen are required for adhesion at any shear rate (26,27). The adherence of platelets is followed by signal transduction which leads to platelet calcium release and ultimately to aggregation. We attempted to test these sequential steps in our in vitro system. To determine whether calcium levels might be affected by exogenous hormones and thereby contribute to thromboembolic disease, $Ca^{2+}_{(i)}$ and release in platelets was studied. The studies described using platelets from women ingesting OC or TAM suggest that one of the effects of ESTR in vivo is to increase platelet $Ca^{2+}_{(i)}$ and that TAM may decrease platelet $Ca^{2+}_{(i)}$. The calcium studies described clearly show that women ingesting OC have significantly higher resting platelet $Ca^{2+}_{(i)}$ and release compared to normal men, normal premenopausal women and women taking TAM. Similarly, incubating platelets with ESTR in vitro from women on OC, normal men, and women, demonstrates an increase platelet $Ca^{2+}_{(i)}$ and calcium release. Our studies agree with those of Morley et al (9). They found that addition of ESTR (10^{-10} - 10^{-6}) to chick and pig granulosa cells was associated with a rapid surge in $Ca^{2+}_{(i)}$. Their results suggest that ESTR has a nongenomic effect, given the nearly instantaneous and transient calcium surge associated with addition of ESTR to the cells. Since platelets are anucleate cells, one would have to postulate that any effect on cellular processes would have to be mediated by a nongenomic mechanism. Collins et al attribute the decreased risk of cardiovascular disease in premenopausal women and postmenopausal women on ESTR replacement to the Ca^{2+} blocking effect of estrogen (28). The increase in $Ca^{2+}_{(i)}$ and release in OC users and in vitro ESTR incubation studies in our studies oppose the proposal of Collins et al (28).

Oral contraceptives contain both, estrogens and progestins. Morley et al found that high concentrations of progesterone were associated with a small rise $Ca^{2+}_{(i)}$ when incubated with chicken and pig granulosa cells (9). They postulate the immediate rise in $Ca^{2+}_{(i)}$ to be an estrogen effect, which differs from the action of progesterone. Progesterone has been found by others to stimulate the influx of Ca^{2+} through membrane channels in target cells (29-33). In vitro incubation of platelets from normal men with progesterone is also associated with an increase in $Ca^{2+}_{(i)}$ and release, however the effect of incubation with ESTR is significantly greater than progesterone.

The effect of OC on Ca^{2+} in vivo can not be attributed solely to ESTR, but the aforementioned studies suggest that ESTR has a major effect and progesterone has a minor effect on on $Ca^{2+}_{(i)}$ and release.

Platelets from women on OC exhibited significantly increased platelet adhesion as well as increased sensitivity of their platelets to aggregating agents when compared to normal control individuals. One could postulate that increased $Ca^{2+}_{(i)}$ and increased calcium release contribute to the increased platelet adhesion and aggregation observed. These findings correlate with the clinical observation that OC users have an increased risk of thrombosis.

For years, it has been debated whether women with breast cancer who take TAM have an increased tendency for thrombotic events. Since breast cancer can be associated with a hypercoagulable state, this issue has been difficult to resolve (34-37). Recently a large study has shown that women with early stage breast cancer do not have an increased risk of thrombosis compared to control women not ingesting TAM (7). Although TAM has partial estrogenic properties, the major mechanism of action is as an antiestrogenic agent. We have found that patients with early stage breast cancer ingesting TAM have decreased platelet adhesion compared to normal control groups (16). The finding that patients taking TAM have decreased platelet $Ca^{2+}_{(i)}$ and calcium release implies that platelet Ca^{2+} levels, adhesion and aggregation are intricately related.

Women ingesting TAM had significantly lower platelet $Ca^{2+}_{(i)}$ and release compared to all other groups studied. Tamoxifen has been postulated to have several mechanisms of action. It completes for estrogen receptors (38-41) and anti-estrogen binding sites (42-47). Others have shown that TAM competes with Ca^{2+} channel antagonist binding sites (48,49). Tamoxifen has been shown to inhibit calmodulin, phospholipid and other Ca^{2+}-activated protein kinases (48). Tamoxifen blocks Ca^{2+} uptake by calmodulin in vitro by interfering with phosphodiesterases (49). Greenberg found that in PC12 murine pheochromocytoma cell lines, TAM inhibited Ca^{2+} influx and competed for Ca^{2+} channel antagonist binding sites (48). Tamoxifen, as well as other protein kinase inhibitors inhibit the uptake of Ca^{2+}, but less than the classical Ca^{2+} channel blockers (50). Others have found that TAM blocks Ca^{2+} entry into smooth muscle (51,52) rather than blocking $Ca^{2+}_{(i)}$ release. Lipton et al studied rat myometrium and aorta and found that TAM did not significantly inhibit the uptake of extracellular Ca^{2+} by depolarization of the cells with potassium (52). In contrast, the Ca^{2+} channel blockers, nifedipine and diltiazem blocked Ca^{2+} influx into the cells. Lipton postulated that TAM might inhibit the slow inward Ca^{2+} entry into smooth muscle cells (51). With TAM concentrations of $<10^{-7}$M, Sartor found that both, fast and slow, inactivating Ca^{2+} currents are blocked by TAM (53). The TAM concentration used by Lipton and Morris was higher (10^{-6}), and perhaps this could account for the different findings. However, all these studies support a role for TAM in inhibiting protein kinase C-dependent processes (52-54). Our study supports the findings from the studies mentioned above, in that patients ingesting TAM had the lowest platelet $Ca^{2+}_{(i)}$ and release compared to all other groups. Certainly one could postulate decreased Ca^{2+} uptake by the platelet and/or inhibition of calmodulin or other

intracellular mechanisms. Unlike ESTR, in vitro incubation of normal platelets from men with TAM did not affect $Ca^{2+}_{(i)}$ or release compared to control. This supports the postulated mechanism that TAM acts at least in part, at non-estrogen receptors. Whether the TAM acts on the platelet itself or on the megakaryocyte is unclear.

As Morley and colleagues postulated (9), if in fact there are receptors on the platelet surface which trigger calcium release from the cell by signal transduction, our findings suggest that estrogen activates, potentiates or may be a ligand for this receptor, whereas TAM might block, inactivate, or down-regulate the receptor. Non-estrogen (steroid) receptors on the platelet surface may indirectly activate the platelet causing a cell signaling reaction which in turn leads to calcium release. Calcium release may be due to other factor(s) not identified in this study, however, given the significant differences between the $Ca^{2+}_{(i)}$ levels in normal individuals and the TAM and OC user groups, estrogen and antiestrogenic agents can likely be implicated to have either a direct or indirect role in calcium modulation in platelets.

Subsequent studies attempted to determine if the ex vivo findings could be reproduced in vitro. Platelets from normal men and women incubated in vitro with ESTR had increased platelet $Ca^{2+}_{(i)}$ and release compared to their own control (unincubated platelets), but these changes did not correlate with platelet adhesion or aggregation. Platelets incubated in vitro with ESTR exhibited decreased adhesion to fibronectin at low shear rate. Since platelet adhesion is the first major step in thrombus formation, this discrepancy between ex vivo and in vitro observations with ESTR may yield some basic information about the mechanisms operative in the adhesion process. As reported by Morley et al, estrogen causes a rapid release of calcium from chicken granulosa cells by a nongenomic action triggered by a signal-generating receptor on the cell surface, and was not blocked by verapamil (9). Calcium release was blocked by verapamil in our study, suggesting that the estrogen-induced $Ca^{2+}_{(i)}$ increase was likely due to the release of $Ca^{2+}_{(i)}$ from internal stores alone and involving slow Ca^{2+} channels (55). Neomycin had an effect similar to verapamil, indicating that the phosphoinositide pathway is probably affected (56). The rapid action of ESTR is probably a direct action of the transduction of a steroid signal from the cell surface.

The discrepancies between the ex vivo and in vitro studies is unclear, but it would be unlikely that addition of hormonal agents to platelets in vitro would completely mimic in vivo conditions. Although doses of ESTR and TAM were calculated to approximate serum levels in vivo, one can not account for drug metabolites, continuous balance between coagulation and fibrinolytic systems, and exposure of megakaryocytes and platelets to continuous levels of hormonal agents. Diluents used for TAM and ESTR, METH and PBS respectively, did not have any discernable adverse effect on the study platelets.

While $Ca^{2+}_{(i)}$ levels were increased by ESTR but not by TAM incubation the platelets did not exhibit increased adhesion or aggregation except when platelets from men were incubated with TAM. This may have several explanations. One is that incubating normal platelets in ESTR may be associated with a rapid release of $Ca^{2+}_{(i)}$ whereas platelets from either OC users or women on TAM may have reached equilibrium.

Women on OC had the greatest adhesion and had increased aggregation (8) while women on TAM had decreased adhesion and seemingly normal platelet aggregation. These observations are in keeping with the incidence of thromboembolic disease seen clinically when each drug is consumed. It may be that additional factors are operative in vivo. The consumption of ESTR may cause an up-regulation of receptor numbers for platelet adhesive glycoproteins whereas when platelets are incubated with ESTR in vitro, ESTR may bind to the platelet and interfere with signal transduction. Platelet membrane glycoproteins have been extensively reviewed by McEver (57). Alternatively, changes induced in coagulation factors by OC use may, in part, be responsible for the increase in adhesion and aggregation observed in these patients. Our tests for adhesion and aggregation used PRP, whereas those measuring $Ca^{2+}_{(i)}$ used washed platelets.

In conclusion, our data show that changes in platelet $Ca^{2+}_{(i)}$ may contribute to platelet adhesion and aggregation. Platelet $Ca^{2+}_{(i)}$ and calcium release correlate with the degree of platelet adhesion at low shear rate, suggesting that these processes are intricately related. Altering the hormonal milieu in vivo by consumption of OC or TAM appears to alter platelet $Ca^{2+}_{(i)}$ and calcium release. The mechanism by which this occurs in the platelet is likely to be a nongenomic mechanism through a non-steroid receptor. Further studies are needed to clarify this mechanism, given the ubiquitous use of hormonal agents.

REFERENCES

1. STADEL, B.V. Oral contraceptives and cardiovascular disease. N. Engl. J. Med. *305*, 612-8; 672-7, 1981.
2. HELMRICH, S.P., ROSENBERG, L., KAUFMAN, D.W., STROM, B. and SHAPIRO, S. Venous thromboembolism in relation to oral contraceptive use. Obstet. Gynecol. *69*, 91-95, 1987.
3. BYAR, D.P. and CORLE, D.K. Hormone therapy for prostate cancer: results of the Veterans Administration Cooperative Urological Research Group studies. In: *NCI Monographs*, No. 7, pp 165-70, Government Printing Office, Washington, D.C. (1988).
4. DeVOOGT, H.J., SMITH, P.H., PAVONE-MACALUSO, M., DePAUW, M. and SUCIU, S. Cardiovascular side effects of diethylstilbestrol, crypterone acetate, medroxyprogesterone acetate and extramustine phosphate used for the treatment of advanced prostatic cancer: results from European Organization for Research on Treatment of Cancer trials 30761 and 30762. J. Urol. *135*, 303-7, 1986.
5. ROGERS, J.S., MURGO, A.J., FONTANA, J.A. and RAICH, P.C. Chemotherapy for breast cancer decreases plasma protein C and protein S. J. Clin. Oncol. *6*, 276-281, 1988.
6. ENCK, R.E. and RIOS, C.N. Tamoxifen treatment of metastatic breast cancer and antithrombin III levels. Cancer *53*, 2607-2609, 1984.
7. FISHER, B., COSTANTINO, J., REDMOND, C., POISSON, R., BOWMAN, D., COUTURE, J., DIMITROV, N.V., WOLMARK, N., WICKERHAN, D.L., FISHER, E.R., MARGOLESE, R., ROBIDOUX, A., SHIBATA, H., TERZ, J., PATERSON, A.H.G., FELDMAN, M.I., FARRAR, W., EVANS, J., LICKLEY, H.L., KETNER, M. and

OTHERS. A randomized trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen receptor-positive tumors. N. Engl. J. Med. *320*, 479-84, 1989.
8. DORES, G.M., MILLER, M.E. and THORPE, S.L. Platelet adhesion at low shear rate: study of a normal population. Thromb. Res. *69*, 173-184, 1993.
9. MORLEY, P., WHITFIELD, J.F., VANDERHYDEN, B.C., TSANG, B.K. and SCHWARTZ J.L. A new, nongenomic estrogen action: the rapid release of intracellular calcium. Endocrinol. *131*, 1305-1312, 1992.
10. BORN, G.V.R. and CROSS, M.J. The aggregation of blood platelets. J. Physiol. *168*, 178-182, 1963.
11. GRYNKIEWICZ, G., POENIE, M. and TSIEN, R.Y. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. *260*, 3440-3450, 1985.
12. TEPEL, M., WISCHNIOWSKI, H. and ZIDEK, W. Erythropoietin induced transmembrane calcium influx in essential hypertension. Life Sci. *51*, 161-7, 1992.
13. RICHARDSON, P.D., MOHAMMED, S.F. and MASON, R.G. Flow chamber studies of platelet adhesion at controlled spatially varied shear rates. Proc. Eur. Soc. Artif. Organs. *4*, 175-179, 1977.
14. BATCHELOR, G.K. *An Introduction to Fluid Dynamics.* Cambridge University Press: Cambridge, UK (1967).
15. KANE, R.L. *Spatio-temporal dynamics of human platelet adhesion in a Hele-Shaw flow chamber.* Master Thesis, Brown University, June 1981.
16. MILLER, M.E., DORES, G.M., THORPE, S.L. and AKERLEY, W.L. Paradoxical influence of estrogenic hormones on platelet-endothelial cell interactions. Thromb. Res. (In press).
17. BONNAR, J. Coagulation effects of oral contraception. Am. J. Obstet. Gynecol. *157*, 1042-8, 1987.
18. BELLER, F.K. and EBERT, C. Effects of oral contraceptives on blood coagulation. A review. Obstet. Gynecol. Surv. *40*, 425-36, 1985.
19. NOTELOVITZ, M. Oral contraception and coagulation. Clin. Obstet. Gynaecol. *28*, 73-83, 1985.
20. MAMMEN, E.F. Oral contraceptives and blood coagulation: a critical review. Am. J. Obstet. Gynecol. *142*, 781-90, 1982.
21. SHEMIN, D., ELNOUR, M., AMARANTES, B., ABUELO, J.G. and CHAZEN, J.A. Oral estrogens decrease bleeding time and improve clinical bleeding in patients with renal failure. Amer. J. Med. *89*, 436-440, 1990.
22. CORVAZIER, E., DUPUY, E., DOSNE, A.M. and MACLOUF, J. Minimal effect of estrogens on endothelial cell growth and production of prostacyclin. Thromb. Res. *34*, 303-310, 1984.
23. HARRISON, R.L. and McKEE, P.A. Estrogen stimulates von Willebrand factor production by cultured endothelial cells. Blood *63*, 657-665, 1984.
24. deGROOT, P. and SIXMA, J.J. Annotation. Platelet adhesion. Br. J. Hematol. *75*, 308-312, 1990.
25. TURITTO, V.T. and BAUMGARTNER, H.R. Initial deposition of platelets and fibrin on vascular surfaces in flowing blood. In: *Hemostasis and Thrombosis*, 3rd ed., J.B. Lippincott Company, pp. 805-822, 1993.
26. HYNES, R.O. The complexity of platelet adhesion to extracellular matrices. Thromb. Haemostasis *66*, 40-43, 1991.

27. SAELMAN, E.U.M., NIEUWENHUIS, H.K., HESE, K.M., deGROOT, P.G., HEIJNEN, H.F.G., SAGE, E.H., WILLIAMS, S., McKEOWN, L., GRALNICK, H.R. and SIXMA, J.J. Platelet adhesion to collagen types I through VIII under conditions of stasis and flow is mediated by GPIa/IIa ($\alpha_2\beta_1$-integrin). Blood *83*, 1244-1250, 1994.
28. COLLINS, P., ROSANO, G.M., JIANG, C., LINDSAY, D., SARREL, P.M. and POOLE-WILSON, P.A. Cardiovascular protection by oestrogen--a calcium antagonist effect? Lancet *341*, 1264-1265, 1993.
29. BLACKMORE, P.F., BEEBE, S.J., DANFORTH, D.R. and ALEXANDER, N. Progesterone and 17α-hydroxyprogesterone: novel stimulators of calcium influx in human sperm. J. Biol. Chem. *265*, 1376-1380, 1990.
30. LIEBERHERR, M. Effects of vitamin D_3 metabolites on cytosolic free calcium in confluent mouse osteoblasts. J. Biol. Chem. *262*, 13168-13173, 1987.
31. CIVITELLI, R., KIM, Y.S., GUNSTEN, S.L., FUJIMORI, A., HUSKEY, M., AVIOLI, L.V. and HRUSKA, K.A. Nongenomic activation of the calcium message system by vitamin D metabolites in osteoblast-like cells. Endocrinology *127*, 2253-2262, 1990.
32. deBOLAND, A.R. and NORMAN, A. Evidence for involvement of protein kinase C and cyclic adenosine 3',5'-monophosphate-dependent protein kinase in the 1,25-dihydroxyvitamin D_3-mediated rapid stimulation of intestinal calcium transport (transcaltachia). Endocrinology *127*, 39-45, 1990.
33. deBOLAND, A.R. and NORMAN, A.W. Influx of extracellular calcium mediates 1,25-dihydroxyvitamin D_3-dependent transcaltachia (the rapid stimulation of duodenal Ca2+ transport). Endocrinology *127*, 2475-2480, 1990.
34. SCHAFER, A.I. The hypercoagulable states. Ann. Intern. Med. *102*, 814-828, 1985.
35. PATTERSON, W.P. and RINGENBERG, Q.S. The pathophysiology of thrombosis in cancer. Semin. Oncol. *17*, 140-146, 1990.
36. LUZZATTO, G. and SCHAFER, A.I. The prethrombotic state in cancer. Semin. Oncol. *17*, 147-159, 1990.
37. TROUSSEAU, A. Phlegmatia alba dolens. In: *Clinique Medicale de l'Hotel-Dieu de Paris*, 2nd ed., Paris, J-B Bailliere, vol. 3, pp. 654-712, (1865).
38. LIPPMAN, M.E., BOLAN, G. and HUFF, K.K. The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long term tissue culture. Cancer Res. *36*, 4595-4601, 1978.
39. COEZY, E., BORGNA, J.L. and ROCHEFORT, H. Tamoxifen and metabolites in MCF-7 cells: correlation between binding to estrogen receptor and inhibition of cell growth. Cancer Res. *42*, 317-323, 1982.
40. REDDEL, R.R., MURPHY, L.C. and SUTHERLAND, R.L. Effects of biologically active metabolites of tamoxifen on the proliferation kinetics of MCF-7 human breast cancer cells in vitro. Cancer Res. *43*, 4618-4624, 1983.
41. OSBORNE, C.K., HOBBS, K. and CLARK, G.M. Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. Cancer Res. *45*, 584-590, 1985.
42. SUTHERLAND, R.L. and MURPHY, L.C. The binding of tamoxifen to human mammary carcinoma cytosol. Eur. J. Cancer *16*, 1141-1148, 1980.
43. SUTHERLAND, R.L., MURPHY, L.C., FOO, M.S., GREEN, M.D., WHYBOURN, A.M. and KROZOWSKI, Z.S. High-affinity anti-oestrogen binding site distinct from the oestrogen receptor. Nature (Lond.) *288*, 273-275, 1980.

44. GULLINO, A. and PASQUALINI, J.R. Heterogeneity of binding sites for tamoxifen and tamoxifen derivatives in estrogen target and nontarget fetal organs of guinea pig. Cancer Res. *42*, 1913-1921, 1982.
45. SUDO, K., MONSMA, F.J., Jr. and KATZENELLENBOGEN, B.S. Antiestrogen-binding sites distinct from the estrogen receptor: subcellular location, ligand specificity, and distribution in tissues of the rat. Endocrinology *112*, 425-434, 1983.
46. CHOUVET, C. and SAEZ, S. High affinity cytosol binding site(s) for antiestrogens in two human breast cancer cell lines and in biopsy specimens devoid of estrogen receptors. J. Steroid biochem. *21*, 755-761, 1984.
47. LAZIER, C.B. Interactions of tamoxifen in the chicken. J. Steroid Biochem. *27*, 877-882, 1987.
48. GREENBERG, D.A., CARPENTER, C.L. and MESSING, R.O. Calcium channel antagonist properties of the antineoplastic antiestrogen tamoxifen in the PC12 neurosecretory cell line. Cancer Res. *47*, 70-74, 1987.
49. LAM, P.H.-Y. Tamoxifen is a calmodulin antagonist in the activation of cAMP phosphodiesterase. Biochem. Biophys. Res. Commun. *118*, 27-32, 1984.
50. MESSING, R.O., CARPENTER, C.L. and GREENBERG, D.A. Mechanisms of calcium channel inhibition by phenytoin: comparison with classical calcium channel antagonists. J. Pharmacol. Exp. Ther. *235*, 407-411, 1985.
51. LIPTON, A. The anti-estrogen tamoxifen is a calcium antagonist in perfused rat mesentery. Cancer. Chemother. Pharmacol. *20*, 125-127, 1987.
52. LIPTON, A. and MORRIS, I.D. Calcium antagonism by the antiestrogen tamoxifen. Cancer Chemother. Pharmacol. *18*, 17-20, 1986.
53. SARTOR, P., VACHER, P., MOLLARD, P. and DUFY, B. Tamoxifen reduces calcium currents in a clonal pituitary cell line. Endocrinology *123*, 534-540, 1988.
54. O'BRIAN, C.A., LISKAMP, R.M., SALOMON, D.H. and WEINSTEIN, I.B. Inhibition of protein kinase C by tamoxifen. Cancer Res. *45*, 2462, 1985.
55. FRISHMAN, W.H. and SONNENBLICK, E.H. Calcium Channel Blockers. In: *The Heart Arteries and Veins*, 7th ed., McGraw-Hill, Inc., pp. 1731-1748 (1990).
56. CARNEY, D.H., SCOTT, D.L., GORDON, E.A. and LaBELLE, E.F. Phosphoinositides in mitogenesis: neomycin inhibits thrombin-stimulated phosphoinositide turnover and initiation of cell proliferation. Cell *42*, 479-488, 1985.
57. McEVER, R.P. The clinical significance of platelet membrane glycoproteins. Hematology/Oncology Clinics of North America *4*, 87-105, 1990.