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Progesterone metabolites rapidly stimulate calcium influx in human platelets by a *src*-dependent pathway

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ABSTRACT

The effects of several steroids and their metabolites were examined for their ability to rapidly alter intracellular free calcium ($[Ca^{2+}]_i$) in the anucleate human platelet. Earlier studies suggested that steroids had direct and rapid non-genomic effects to alter platelet physiology. The rationale for performing this study was to investigate the signal transduction events being activated by steroids. Super-physiologic concentrations (1.0–10.0 μM) of β-estradiol and several estradiol metabolites and analogs potentiated (approximately twofold) the action of thrombin to elevate $[Ca^{2+}]_i$ in platelets, whereas 10.0 μM progesterone inhibited the action of thrombin by 10–15%. Progesterone and β-estradiol by themselves did not affect $[Ca^{2+}]_i$. Progesterone metabolites can achieve high blood concentrations. Some progesterone metabolites, particularly those in the β-conformation, were potent stimulators of Ca^{2+} influx and intracellular Ca^{2+} mobilization in platelets. They activated phospholipase C because their ability to increase $[Ca^{2+}]_i$ was inhibited by the phospholipase C inhibitor U-73122. The ability of pregnanediol and collagen to increase $[Ca^{2+}]_i$ was inhibited by the *src* tyrosine kinase inhibitor PP1, whereas the actions of thrombin and thapsigargin to increase $[Ca^{2+}]_i$ were not affected by PP1. The effects of progesterone metabolites to increase $[Ca^{2+}]_i$ were observed with concentrations as low as 0.1 μM. Pregnanolone synergized with thrombin to increase $[Ca^{2+}]_i$. It is hypothesized that human platelets possess receptors for progesterone metabolites. These receptors when stimulated will activate platelets by causing a rapid increase in $[Ca^{2+}]_i$. Pregnanolone, isopregnanediol and pregnanediol were the most effective stimulators of this newly identified *src*-dependent signal transduction system in platelets. Progesterone metabolites may regulate platelet aggregation and hence thrombosis *in vivo*.

1. Introduction

Two studies demonstrated that platelets respond to progesterone and β-estradiol in a rapid non-genomic manner *in vitro* [1,2]. The study of Raman et al. [2] showed that a 30-min pretreatment with either β-estradiol (10 μM) or progesterone (10 μM) attenuated (up to 50%) the effect of a high concentration of vasopressin (0.1 μM) to increase intracellular free calcium ($[Ca^{2+}]_i$) in human platelets from either healthy males or females. The study of Miller et al. [1] showed that β-estradiol (10 ng/ml) and progesterone (10 μM) increased $[Ca^{2+}]_i$ in platelets from men and women after 30 min (by approximately 100%). However no direct effects of β-estradiol or progesterone on $[Ca^{2+}]_i$ were observed in the present study. The effect of thrombin to increase $[Ca^{2+}]_i$ was potentiated by β-estradiol in platelets from men and women [1]. Neomycin, a

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non-specific phospholipase C inhibitor, attenuated the effect of β-estradiol to elevate $[Ca^{2+}]_i$ as did the L-type calcium channel blocker verapamil at a very high concentration (50 μM). These results suggested that that β-estradiol increased phosphoinositide turnover and that L-type calcium channels were activated by the steroid.

The first extensive review of non-genomic actions of steroids was published by Duval et al. [3]. In the 1990s the field of non-genomic actions of steroids expanded greatly and has been reviewed recently [4–13].

Platelets play an essential role in hemostasis (e.g. Ref. [14]) and the inflammatory response [15]. They are activated by many factors that cause them to undergo a major transformation known as aggregation and shedding of microvesicles. An increase in $[Ca^{2+}]_i$ is an important component for these events to occur. As in most non-excitable cells the increase in platelet $[Ca^{2+}]_i$ induced by various agonists involves both influx of extracellular Ca^{2+} and mobilization of intracellular Ca^{2+} (from the dense tubular system). Most platelet agonists (e.g. ADP, thrombin and thromboxane A_2) activate phospholipase C-β_1 (PLC) *via* a heterotrimeric GTP-binding protein [16], while collagen activates PLC-γ_2 [17–20].

The mechanism of Ca^{2+} influx in platelets is less well understood. Some studies have suggested the involvement of voltage-gated Ca^{2+} channels; however most evidence is against this. Depolarization of platelet plasma membrane with high 60 mM K^+ does not increase $[Ca^{2+}]_i$ and the Ca^{2+} channel blockers verapamil, diltiazem and nifedipine do not inhibit thrombin-induced increases in $[Ca^{2+}]_i$ (P.F.B. unpublished observations and [21,22]). Therefore Ca^{2+} enters the platelet following thrombin stimulation independent of membrane depolarization. The channel that is activated by a variety of hormones such as thrombin has the following features. It is not inhibited by voltage-gated Ca^{2+} channel antagonists and is relatively insensitive to membrane depolarization. The channel was activated when intracellular Ca^{2+} was mobilized by inositol 1,4,5-P_3, and was also activated by Ca^{2+} store depletion with agents, such as thapsigargin [23], thus it has the properties of a store-operated calcium channel (SOCC). The channel can be blocked by Ni^{2+} and La^{3+} ions, SK&F-96365 and several azole antifungal agents [16]. Recent evidence supports an intimate contact between the intracellular inositol 1,4,5-trisphosphate receptor/channel in the endoplasmic reticulum and the transient receptor potential (TRP) located in the plasma membrane. TRP channel may be the long sought after SOCC, alternatively SOCC may be Orai1 that is regulated by STIM1 (for references see [24]). In platelets, evidence supports this conformational coupling mechanism, since co-immunoprecipitation experiments show a coupling of endogenously expressed hTrp1 with type II IP_3 receptors when intracellular Ca^{2+} stores are depleted [25,26].

The purpose of this present study was to investigate the mechanism by which various steroids rapidly regulate $[Ca^{2+}]_i$ in human platelets. When members of other steroid classes were examined to gain insight into the specificity of the effects, it was discovered that several progesterone metabolites were very active at rapidly increasing $[Ca^{2+}]_i$ in platelets, suggesting that non-genomic steroid receptors exist on platelets. This finding of a novel platelet activating system that was stimulated by progesterone metabolites may result in identifying new pharmacological approaches, such as steroid analogs, to regulate platelet aggregation and hence thrombosis.

2. Experimental

2.1. Reagents and material sources

The following were from Sigma Chemical Co.: β-estradiol (17β-estradiol), progesterone (4-pregnene-3,20-dione), 4-pregnen-20α-ol-3-one (20α-hydroxyprogesterone), 4-pregnen-20β-ol-3-one (20β-hydroxyprogesterone), 5β-pregnane-3,20-dione (5β-dihydroxyprogesterone), 5α-pregnane-3,20-dione (5α-dihydroprogesterone), 5β-pregnan-3α-ol-20-one (pregnanolone), 5α-pregnan-3α-ol-20-one (allopregnanolone), 5α-pregnan-3β-ol-20-one (isopregnanolone), 5β-pregnane-3α,20α-diol (pregnanediol), 5α-pregnane-3α,20α-diol (allopregnanediol), 5α-pregnane-3β,20α-diol (isopregnanediol), 5β-pregnan-3β-ol-20-one (epipregnanolone), 5β-pregnan-3α, 17, 20α-triol (pregnanetriol), 5-pregnen-3β-ol-20-one (pregnenolone), 4-androsten-3, 17-dione (androstenedione), 5-androsten-3β-ol-17-one (dehydroisoandrosterone), testosterone, β-estrdiol-3-benzoate, β-estrdiol-3,17-dipropionate, estriol-3-methyl ether, estrone-3-methyl ether, ethyl-estradiol-3-methyl ether, estriol, estrone, androsterone, α-estradiol, estrone-3-sulfate, cortisone, cortisol, corticosterone, 17-ethylnyl estradiol, deoxycorticosterone, diethylstilbestrol, thrombin, dimethylsulfoxide (Me_2SO) and ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). The following were from Steraloids, Inc.: 5α-pregnane-3α,21-diol-20-one (allo tetrahydrodeoxycorticosterone, allo THDOC) and 5β-pregnane-3α,21-diol-20-one (tetrahydrodeoxycorticosterone, THDOC). The following were from Calbiochem: thapsigargin, ionomycin, U-73122 and U-73343. Fura-2/AM was from Molecular Probes. The anti-estrogen MER-25 was from Merrell Dow Research Institute, Cincinnati, OH. Other chemicals were from Fisher Scientific and Sigma Chemical Co. Collagen was from CHRONO-LOG Corp. Havertown, PA. The *src* inhibitor 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1) and PP3 (non-functional analog of PP1) were from Alexis Biochemicals, San Diego, CA. Phenylarsine oxide was from Aldrich Chemical Co., Milwaukee, WI.

2.2. Blood donors and platelet preparation

All donors were healthy volunteers (ages 20–40) who had not consumed any medication known to affect platelet function for at least 10 days prior to the study. The Institutional Review Board at Eastern Virginia Medical School has approved this protocol; subjects gave informed consent for the study. Venous blood was collected into 1/10 volume of ACD (74.8 mM sodium citrate, 38.1 mM citric acid and 123 mM dextrose pH 6.4) (Baxter Healthcare Corp.). The blood was centrifuged at $250 \times g$ for 10 min at room temperature to obtain platelet rich plasma (PRP). The PRP was centrifuged at $550 \times g$ for 12 min to sediment the platelets. The platelets were then re-suspended in a modified Tyrodes physiological salt solution (NaCl, 145.0 mM; KCl, 4.0 mM; $MgSO_4$, 1.0 mM; Na_2HPO_4,