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Phorbol 12,13-dibutyrate binding to intact human platelets

The role of cytosolic free Ca^{2+}

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The role of Ca^{2+} was examined in regulating the binding of phorbol 12,13-dibutyrate (PdBu) to intact human platelets. Alterations in the cytosolic free Ca^{2+} concn. ($[Ca^{2+}]_i$), but not extracellular Ca^{2+}, substantially influenced the binding parameters of the phorbol ester. Ca^{2+}-depleted platelets demonstrated a significant decline in the maximal binding capacity ($B_{max.}$), an increase in equilibrium dissociation constant (K_d) and a decrease in the Hill coefficient (h), suggesting the presence of Ca^{2+}-sensitive and Ca^{2+}-insensitive populations of PdBu-binding sites. In 1 mM-Ca^{2+} buffer, thrombin (0.1 NIH unit/ml) and ionomycin (0.5 μM) evoked a rise in $[Ca^{2+}]_i$ to approx. 300–500 nM, associated with a significant decline in K_d, but without an apparent effect on $B_{max.}$. No effect of thrombin was observed on PdBu binding in Ca^{2+}-depleted platelets. Inhibition of protein kinase C (PKC) by H7 was associated with a greater thrombin-evoked $[Ca^{2+}]_i$ transient and a decline in K_d. Staurosporine also decreased the K_d for PdBu binding. We propose that this effect of the PKC inhibitors on the K_d was also $[Ca^{2+}]_i$-dependent. These observations in intact platelets indicate that the primary role of agonist- or non-agonist-induced rise in $[Ca^{2+}]_i$ is to increase the affinity of PKC for PdBu and, presumably, endogenous diacylglycerol. However, in itself a rise in $[Ca^{2+}]_i$ does not increase the $B_{max.}$ for PdBu binding.

INTRODUCTION

Protein kinase C (PKC) plays a central role in multiple cellular processes. Agonist-mediated activation of this enzyme usually involves the stimulation of phospholipase C. The cellular messengers, inositol 1,4,5-trisphosphate and diacylglycerol (DAG), and the rise in the cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) that result from this stimulation initiate a chain of events, including activation of PKC. This process is preceded by the translocation of the enzyme from the cytosol to the plasma membrane [1,2]. The mechanisms responsible for PKC translocation are not fully understood; however, previous reports suggest that, at least in a sub-population of PKC, they depend on a rise in $[Ca^{2+}]_i$ [2,3]. Phorbol esters have been commonly used to probe PKC because they by-pass the phosphatidylinositol pathway and activate the enzyme by interacting with its DAG site [1,4–8]. Moreover, [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PdBu) binds to receptors that are almost exclusively located in plasma-membrane fractions [9,10]. Such specific [^{3}H]PdBu-binding sites are also present in platelets [11,12]. The purpose of the present work was to study [^{3}H]PdBu binding to intact platelets in order to gain further insight into the mechanisms governing PKC regulation and the dependence of these processes on $[Ca^{2+}]_i$.

METHODS

Platelet preparations

Platelets were isolated as previously described [13]. Briefly, blood was drawn from normal subjects on no medications, including aspirin; 50 ml of blood was drawn from each subject into acid dextrose buffer (20:1, v/v), which included (mM): sodium citrate 14, citric acid 11.8 and dextrose 18 (final pH 6.5). Platelets were studied within 4 h after blood drawing. Blood was centrifuged at 200 g for 10 min at room temperature. The platelet-rich plasma was centrifuged at 1000 g for 10 min, and cells were washed three times (by centrifugation at 1000 g for 10 min) with Hepes-buffered solution (HBS) containing (mM) NaCl 140, KCl 5, glucose 10, Hepes 10 (pH 7.4) plus EGTA 0.2. EGTA was omitted from the third washing; this washing also included 0.1 % fatty-acid-free BSA. Platelets were counted in a Coulter counter (ZBI).

[^{3}H]PdBu binding kinetics

Unless otherwise indicated, platelets were suspended in HBS plus 1 mM-$CaCl_2$ and 0.1 % BSA at a concentration of $(2–3) \times 10^7$ platelets/ml. In some experiments we studied Ca^{2+}-depleted platelets. Ca^{2+}-depletion was performed by treatment of the platelets with EGTA and quin 2-AM [13]. Platelets were preincubated at room temperature for the indicated time intervals with or without various agents and agonists. For [^{3}H]PdBu binding, 220 μl of the platelet suspension was mixed in an Eppendorf micro-centrifuge tube with 30 μl of [^{3}H]PdBu (sp. radioactivity 13.2 Ci/mmol; NET-692; NEN Research Products, Wilmington, DE, U.S.A.) and unlabelled PdBu (final concn. 10 nM-[^{3}H]PdBu and up to 320 nM of unlabelled PdBu). To minimize the possibility of internalization of PdBu, all binding experiments were performed at 0 °C (on ice) with the various agonists or probes or with the vehicles. In initial studies we showed that in control platelets the specific binding of [^{3}H]PdBu reached a plateau within 15 min of incubation, whereas in Ca^{2+}-depleted platelets it reached a plateau within 45 min of incubation. Thus binding experiments for control platelets were carried out by incubation for 15 min and for Ca^{2+}-depleted platelets by incubation for 45 min. In this regard, our observation that PdBu binding in control platelets reached a plateau in 15 min differs from that by Goodwin & Weinberg [11], who demonstrated that the [^{3}H]PdBu binding to human platelets reached a plateau at approx. 120 min of incubation at 4 °C. The reason for the difference between the two studies is not clear. At the termination of the binding

Abbreviations used: PdBu, phorbol 12,13-dibutyrate; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concn.; PKC, protein kinase C; DAG, diacylglycerol; HBS, Hepes-buffered solution; PMA, phorbol 12-myristate 13-acetate; OAG, *sn*-1-oleoyl-2-acetylglycerol.

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experiment, samples were rapidly filtered on Whatman GF/C filters (cat. no. 1822025; Whatman International, Maidstone, Kent, U.K.) presoaked with modified Tyrode buffer containing (mM): NaCl 130, KCl 5, NaH_2PO_4 1, $NaHCO_3$ 24, glucose 10, sucrose 12.5 and EDTA 4, plus 0.1 % BSA. Cells were rapidly washed with 4×1 ml of the same ice-cold buffer. Filters were allowed to dry and counted for radioactivity in ECOLUME (ICN, Costa Mesa, CA, U.S.A.). Non-specific binding was determined in the presence of 10 μM-PdBu, and comprised 7–9 % of total binding. There were no effects of vehicles used to dissolve some of the agents (dimethyl sulphoxide 0.2 %, ethanol 0.1 %) on the [^{3}H]PdBu binding parameters. Since the binding experiments were performed on ice, results reflect the effect of treatments, superimposed on potential chilling-evoked changes in platelet structure and function.

$[Ca^{2+}]_i$ measurements

Platelets were incubated with 5 μM fura 2-AM (Molecular Probes, Eugene, OR, U.S.A.) for 30 min at 37 °C in HBS with 1 mM-$CaCl_2$ or in HBS without $CaCl_2$, plus 0.3 mM-EGTA and 200 μM quin 2-AM (for Ca^{2+}-depleted platelets). Basal $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ after treatment with agonists or other experimental perturbations were monitored under constant stirring in a SPEX Flurolog II spectrofluorimeter (model CM-3). Excitation wavelengths were set at 340/380 nm and emission at 505 nm. Autofluorescence of cells and various reagents was subtracted from the total fluorescence.

Data analysis

Since the platelet-binding parameters from different subjects demonstrated inter-individual variability, experimental protocols depicted in each Figure and in Table 1 were performed with platelets from the same individuals. Platelets from at least four subjects were used for each of these protocols; every assay was run in duplicate. Computation of the binding parameters was performed as previously described [14], by using the equation:

$$B = B_{max.} \times \{L/K_d \times [1 + (i/K_d)^h] + L\}$$

where $B_{max.}$ = maximal binding capacity (expressed as molecules or specific binding sites per platelet), B = binding capacity, L = concentration of [^{3}H]PdBu, K_d = equilibrium dissociation constant, i = concentration of unlabelled PdBu, and h = the Hill coefficient. Data analysis utilized a one-way analysis of variance (ANOVA) or the Student t test. Data are presented as means ± S.E.M.

RESULTS

Fig. 1 depicts the displacement of [^{3}H]PdBu by unlabelled PdBu, phorbol 12-myristate 13-acetate (PMA) and *sn*-1-oleoyl-2-acetylglycerol (OAG). Results demonstrate that both PMA and OAG can displace [^{3}H]PdBu from its receptor with respective K_i values of 5.48 nM and 36.3 μM.

The temporal change in thrombin-evoked alterations of PdBu binding was explored by preincubation of platelets with 0.1 NIH unit of thrombin/ml in 1 mM-Ca^{2+}/HBS for 30 s, 1 min and 5 min. Preincubation for 30 s and 1 min did not significantly alter [^{3}H]PdBu binding (result not shown). The effect of thrombin was expressed only after preincubation for 5 min (Fig. 2), as shown by the significantly decreased K_d for PdBu, from 47.2 ± 4.6 to 32.9 ± 6.1 nM. Thrombin treatment also resulted in a significant decline in the Hill coefficient (h), from 1.16 ± 0.06 to 0.994 ± 0.043. There was no effect of thrombin on the $B_{max.}$.

Thrombin raised $[Ca^{2+}]_i$ from basal level of 61 ± 18.9 nM to a peak of 386 ± 74.3 nM. At 300 s after thrombin treatment, $[Ca^{2+}]_i$ level was still at 114 ± 34 nM (P = 0.0583 compared with basal). To examine further the role of a rise in $[Ca^{2+}]_i$ in the thrombin-evoked alterations in PdBu binding, experiments were performed in Ca^{2+}-depleted platelets (protocol 1, Table 1). Ca^{2+} depletion resulted in: (a) a fall in $B_{max.}$ from $(1.96 \pm 0.18) \times 10^5$ to $(1.23 \pm 0.11) \times 10^5$ sites/platelet, (b) a decline in h from 1.16 ± 0.07 to 0.87 ± 0.06, and (c) a decrease in the affinity of PdBu, as expressed by a rise in the K_d from 51.7 ± 8.2 to 157.2 ± 34.5 nM. Thrombin treatment of Ca^{2+}-depleted platelets produced no further effect on the $B_{max.}$ [$(1.06 \pm 0.16) \times 10^5$ sites/platelet] or h (0.92 ± 0.08), but it did partially reverse the diminished affinity to PdBu produced by Ca^{2+} depletion (K_d = 96.3 ± 26.5 nM).

To explore further the role of extracellular Ca^{2+} and $[Ca^{2+}]_i$ in PdBu binding, platelets were preincubated for 5 min with 0.5 μM-ionomycin and underwent [^{3}H]PdBu binding with the ionophore in 1 mM-Ca^{2+}/HBS. In other platelets, not treated with ionomycin, binding assays were performed in Ca^{2+}-free HBS (plus 0.3 mM-EGTA). In Ca^{2+}-containing medium, ionomycin significantly decreased the K_d from 36.9 ± 2.5 to 20.3 ± 2.9 nM, but had

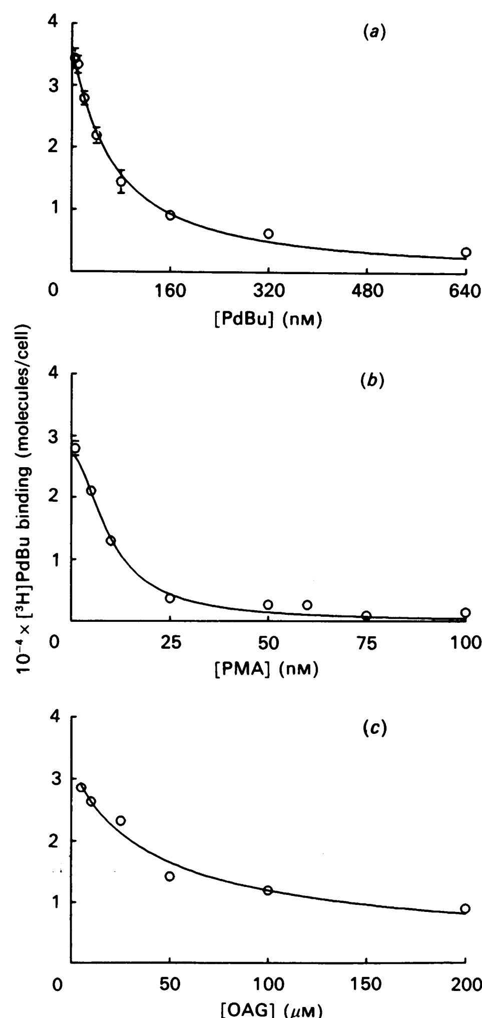

Fig. 1. Displacement of [^{3}H]PdBu by unlabelled PdBu (a), PMA (b) and OAG (c)

Lines in Figs. 1 and 2 represent the fit of the data to the model described in the Methods section. Lack of vertical bars indicates that the S.E.M. is within the symbol.

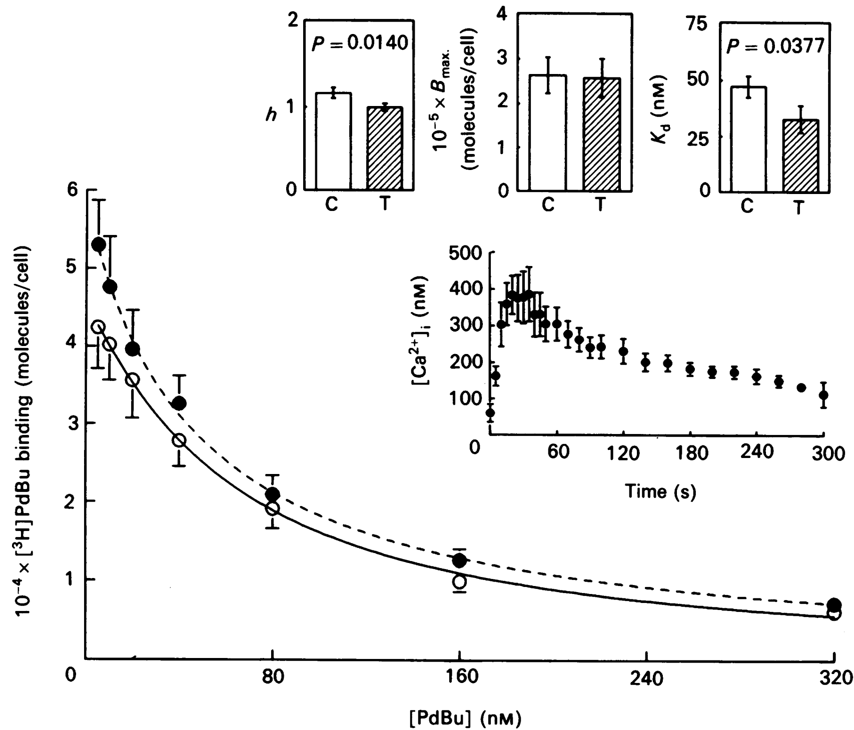

Fig. 2. Effect of thrombin on PdBu binding for control platelets (○, ——) and thrombin-treated platelets (●, ----)

Platelets were preincubated at room temperature for 5 min before institution of the binding experiment. The binding parameters are derived from the overall profile of the displacement of [³H]PdBu by unlabelled PdBu, according to the model described in the Methods section. Upper insets depict h, $B_{max.}$ and K_d for control (C) and thrombin-treated (T) platelets, derived from the main graph; P refers to significance by Student's t test. Lower inset shows the $[Ca^{2+}]_i$ profile in response to 0.1 NIH unit/ml of thrombin added at zero time.

Table 1. Platelet PdBu-binding parameters

Abbreviations: D, depletion; T, thrombin. P was measured by ANOVA for protocols 1–3 and by Student's t test for protocol 4; NS, not significant.

Protocol no.	Conditions	$10^{-5} \times B_{max.}$ (molecules/platelet)	K_d (nM)	h
1	Basal	1.96±0.18	51.7±8.2	1.16±0.07
	Ca_i-D	1.23±0.11	157.2±34.5	0.87±0.06
	Ca_i-D+T	1.06±0.16	96.3±26.5	0.92±0.08
	P	0.0032	0.0393	0.0310
2	Basal	1.64±0.06	36.9±2.5	0.97±0.04
	Ionomycin	1.80±0.15	20.3±2.9	1.04±0.03
	Ca^{2+}-free HBS	1.44±0.08	37.1±5.1	0.96±0.07
	P	NS	0.0108	NS
3	Basal	1.74±0.13	43.0±4.9	1.05±0.03
	H7	1.74±0.12	26.2±4.3	0.97±0.02
	H7+T	2.43±0.18	29.1±3.5	1.06±0.05
	P	0.0036	0.0286	NS
4	Basal	1.55±0.13	52.1±9.1	1.08±0.05
	Staurosporine	1.69±0.32	15.6±6.7	1.01±0.06
	P	NS	0.0060	NS

no effect on the $B_{max.}$ (protocol 2, Table 1). There was no effect of extracellular Ca^{2+} on PdBu binding parameters, inasmuch as the removal of Ca^{2+} from the extracellular medium did not alter the binding. $[Ca^{2+}]_i$ after treatment with ionomycin rose from 57±8.4 nM to a peak of 441±51 nM at 140 s. At 300 s, the $[Ca^{2+}]_i$ was 374±46 nM. These are concentrations within the physiological range of peak $[Ca^{2+}]_i$ after platelet stimulation with a variety of agonists.

Since the effect of $[Ca^{2+}]_i$ on [³H]PdBu binding may be exerted by the Ca^{2+}/calmodulin system, binding experiments were performed after 5 min preincubation in the presence or absence of the calmodulin inhibitor R24571 [15]. At 0.5 μM concentration, R24571 did not alter [³H]PdBu binding parameters and did not change the $[Ca^{2+}]_i$ levels. R24571 did not prevent the ionomycin-induced decrease in K_d for [³H]PdBu (result not shown).

The effects of the PKC inhibitor H7 (200 μM) on PdBu binding were then explored [16]. Preincubation for 5 min with subsequent binding in the presence of H7 lowered the K_d from 43.0±4.9 to 26.2±4.3 nM and had no effect on the $B_{max.}$ and h (protocol 3, Table 1). Exposure of H7-treated platelets to thrombin resulted in no further effect on the K_d (29.1±3.5 nM), but it increased the $B_{max.}$ from the control of $(1.74\pm0.12)\times10^5$ to $(2.43\pm0.18)\times10^5$ sites/platelet. Pretreatment of platelets with H7 had no apparent effect on basal $[Ca^{2+}]_i$. However, the peak $[Ca^{2+}]_i$ transient (534±41 nM) was significantly higher in H7-treated platelets ($P = 0.0474$) than in platelets unexposed to H7 (411±11 nM). At 300 s after thrombin treatment, the Ca^{2+} levels in H7-treated cells were 123±16 nM, compared with 95.6±6.8 nM in platelets unexposed to H7. That the effect of H7 on the K_d is not unique was demonstrated by preincubation of platelets for 5 min and subsequent PdBu binding in the presence of another PKC inhibitor, staurosporine [17–19] (protocol 4, Table 1). Platelets treated with staurosporine (5 μM) exhibited a decline of K_d from 52.1±9.1 to 15.6±6.7 nM, with no alterations in the $B_{max.}$ and h values.

DISCUSSION

The particulate (plasma-membrane) PKC is in a dynamic equilibrium with its soluble apoenzyme, the cytosolic PKC. To a large extent this equilibrium is regulated by $[Ca^{2+}]_i$ and DAG. Studies using cellular fractions or purified PKC preparations

demonstrated that both agents act synergistically to incorporate the cytosolic PKC into the plasma membrane [2,20–22]. When Ca^{2+} concentration is relatively low, PKC can still be activated, as the dose–response curve for activation of PKC shifts to lower Ca^{2+} concentrations [23,24]. Moreover, PKC comprises Ca^{2+}-dependent and Ca^{2+}-independent sub-populations that can be discerned by PdBu binding [25].

Binding of [^{3}H]PdBu to intact cells depends on several factors, including the density of PKC units, the level of $[Ca^{2+}]_i$ and DAG in the plasma membrane, as well as the affinity of the phorbol ester to the DAG-binding site. The DAG/$[Ca^{2+}]_i$ ratio may also influence parameters of [^{3}H]PdBu binding. It is clear, however, that extracellular Ca^{2+} does not play a direct role in the binding of the phorbol ester to intact platelets.

From observations in intact platelets, Siess & Lapetina [3] concluded that an increase in $[Ca^{2+}]_i$ is more important for the translocation of PKC than the formation of DAG, inasmuch as a rise in $[Ca^{2+}]_i$ 'primes' the PKC for activation by DAG or phorbol esters in the plasma membrane. This conclusion originated from observations that [^{3}H]PdBu binding to human platelets was enhanced both by agonists that activate phospholipase C and mobilize Ca^{2+}, and by Ca^{2+} ionophores; the non-agonist-mediated rise in $[Ca^{2+}]_i$ did not involve phospholipase C stimulation and the consequent increase in the formation of DAG. However, [^{3}H]PdBu binding to intact platelets was performed by these investigators at 20 nM of the phorbol ester; this concentration was insufficient to saturate the binding sites. Thus increased [^{3}H]PdBu binding could relate not only to increased density of membrane-bound PKC receptors but also to increased affinity of the phorbol ester for the binding site. The results of our experiments show that this may well be the case.

Ionomycin-evoked rise in $[Ca^{2+}]_i$ had no effect on the $B_{max.}$ for [^{3}H]PdBu, but it increased the affinity of PdBu to the binding sites. Such an observation agrees with that by May *et al.* [26] in intact HL-60 human leukaemic cells. Thrombin also increased the affinity of [^{3}H]PdBu for its binding sites, and in itself it did not exert an apparent effect on the $B_{max.}$. The thrombin-evoked translocation of PKC to the plasma membrane also entails occupancy of the DAG sites by endogenously produced DAG, resulting in a heterogeneous population of sites available for PdBu binding. This concept is supported by the thrombin-evoked decline in h, suggesting the existence of more than a single population of receptors for [^{3}H]PdBu.

That $[Ca^{2+}]_i$ plays a pivotal role in shaping characteristics of [^{3}H]PdBu binding to intact platelets is further demonstrated by experiments performed in Ca^{2+}-depleted platelets. These cells showed significantly diminished $B_{max.}$ and affinity to the phorbol ester. Ca^{2+} depletion also resulted in a significant decline in the h value, thereby suggesting the presence of Ca^{2+}-dependent and Ca^{2+}-independent PdBu receptors [25]. However, in itself a rise in $[Ca^{2+}]_i$ cannot explain the effect of thrombin on the affinity of the binding site to PdBu. In contrast with the ionomycin-induced rise in $[Ca^{2+}]_i$, which was sustained, the thrombin-evoked increase in $[Ca^{2+}]_i$ reached its peak at approx. 30 s after treatment, and thereafter $[Ca^{2+}]_i$ rapidly declined (Fig. 2). In fact, no effect of thrombin on the binding parameters was demonstrated at the height of the $[Ca^{2+}]_i$ response, when the binding experiments were initiated 30 s and 60 s after exposure to thrombin. When binding experiments were started after 5 min of exposure to thrombin, the $[Ca^{2+}]_i$ level was only $\sim$ 50 nM above basal $[Ca^{2+}]_i$. Thus, although $[Ca^{2+}]_i$ is involved in PdBu binding, other factors are also important in shaping the thrombin-evoked alterations in the binding parameters. This is further demonstrated by the fact that thrombin decreased the K_d for PdBu binding in Ca^{2+}-depleted platelets.

The calmodulin antagonist R24571 did not alter [^{3}H]PdBu binding, suggesting that the calmodulin system is not involved in Ca^{2+}-induced alteration in the binding parameters. Wolf *et al.* [2] have reached a similar conclusion. Other calmodulin antagonists [16,27,28] probably exert their effect because of a direct action on PKC rather than by their anti-calmodulin action.

Staurosporine and H7 are potent inhibitors of PKC [16–19]. However, staurosporine does not interact with the DAG site in partially purified preparations of the enzyme [18,29]. Our work demonstrates that both H7 and staurosporine in fact increase the affinity of the site to PdBu. The reason for this finding is not clear. We propose that, in contrast with experiments using PKC preparations or disrupted cells, H7 and staurosporine increase the affinity of PdBu in intact platelets through a Ca^{2+}-dependent mechanism. It is well established that PKC functions in a negative feedback designed to lower $[Ca^{2+}]_i$ through inhibition of PKC, stimulation of Ca^{2+}-ATPase and modifications of other cellular functions [19,30–34]. Thus inhibition of PKC would result in elevated $[Ca^{2+}]_i$. Such an increase may not be seen under basal conditions, but it is easily demonstrated in the stimulated state [13], as was also shown in the present work. It is thus possible that, under basal conditions, inhibition of PKC is not reflected in the overall $[Ca^{2+}]_i$, but only in the Ca^{2+} level at the sub-membrane domain, where a rise in Ca^{2+} would increase the affinity to PdBu. This notion is supported by observations by Wolf & Baggiolini [35], who demonstrated that staurosporine promoted the association of purified PKC with inside-out vesicles from erythrocyte membranes. This effect was Ca^{2+}-dependent and was shown at Ca^{2+} concentrations that are found in resting cells. It is noteworthy that in our study we could show increased $B_{max.}$ only in platelets treated with both H7 and thrombin, suggesting that the combination of both factors can increase the density of PdBu receptors in intact platelets. The specific mechanisms for this effect are not clear.

In conclusion, $[Ca^{2+}]_i$ plays a pivotal role in [^{3}H]PdBu binding to intact platelets; elevation of $[Ca^{2+}]_i$ results in increased affinity of the binding sites, presumably PKC units, for the phorbol ester. The binding sites appear to comprise sub-populations that are Ca^{2+}-sensitive and Ca^{2+}-insensitive, and possibly other types of receptors. Although thrombin-evoked alterations in PdBu binding depend on $[Ca^{2+}]_i$, other factors may also play a role in this process. Finally, uncertainty regarding the $B_{max.}$ for [^{3}H]PdBu in intact cells exists because of endogenous DAG, and possibly the degree of accessibility of PdBu to the DAG site within the plasma membrane under different experimental conditions.

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