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Research Article

Pharmacological Characterization of Inositol 1,4,5-tris Phosphate Receptors in Human Platelet Membranes

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The phosphatidylinositol (PI) hydrolysis signaling system has been shown to be altered in platelets of depressed and schizophrenic subjects. Inositol (1,4,5) trisphosphate ($Ins(1,4,5)P_3$), an integral component of the PI signaling system, mobilizes Ca^{2+} by activating $Ins(1,4,5)P_3$ receptors. To eventually investigate the role of $Ins(1,4,5)P_3$ receptors in depression and other mental disorders, we characterized $[^3H]Ins(1,4,5)P_3$ binding sites in crude platelet membranes prepared from small amounts of blood obtained from healthy human control subjects. We found a single, saturable binding site for $[^3H]Ins(1,4,5)P_3$ to crude platelet membranes, which is time dependent and modulated by pH, inositol phosphates, and heparin. Since cyclic adenosine monophosphate (cAMP) and Ca^{2+} have been shown to be important modulators in $Ins(1,4,5)P_3$ receptors, in the present study we also determined the effects of various concentrations of $CaCI_2$ and forskolin on $Ins(1,4,5)P_3$ binding to platelet membranes. $CaCI_2$ modulated $[^3H]Ins(1,4,5)P_3$ binding sites in a biphasic manner: at lower concentrations it inhibited $[^3H]Ins(1,4,5)P_3$ binding, whereas at higher concentrations, it stimulated $[^3H]Ins(1,4,5)P_3$ binding. On the other hand, forskolin inhibited $[^3H]Ins(1,4,5)P_3$ binding. Our results thus suggest that the pharmacological characteristics of $[^3H]Ins(1,4,5)P_3$ binding to crude platelet membranes are similar to that of $Ins(1,4,5)P_3$ receptors; and that both Ca^{2+} and cAMP modulate $[^3H]Ins(1,4,5)P_3$ binding in crude platelet membranes.

1. Introduction

Agonist-stimulated activation of cell surface receptors, such as $5HT_{2A}$, $5HT_{2C}$, α adrenergic, and muscarinic receptors, leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate by stimulating phospholipase C (PLC) and subsequently generating two second messengers: diacylglycerol (DG) and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$). DG activates protein kinase C (PKC) [1], while $Ins(1,4,5)\ P_3$ triggers the release of Ca^{2+} from intracellular sources by interacting with $Ins(1,4,5)P_3$ receptors [2–4]. $Ins(1,4,5)P_3$ receptors have been identified and characterized in both central and peripheral tissues, such as the brain [5], the hepatic plasma membranes [6], smooth muscle cells [7, 8], rat cerebral and bovine adrenocortical membranes [9], and the rat cerebellum [10]. Cloning studies in various tissues show the existence of three types (I, II, and III) of $Ins(1,4,5)P_3$ receptors [11–13]. All are believed to act as Ca^{2+} channels [4, 14].

Similar to other cell types, $Ins(1,4,5)P_3$ in platelets also functions as a second messenger and mobilizes calcium (Ca^{2+}) by activating the $Ins(1,4,5)P_3$ receptor site [15, 16], which plays an important role in platelet responses involved in homeostasis and thrombosis. Platelets offer a suitable peripheral model for studying abnormalities in neurotransmitter receptors and receptor-mediated second messenger systems such as adenylyl cyclase-cyclic adenosine monophosphate (cAMP) and the phosphatidyl inositol (PI) hydrolysis signaling system. Some reports indicate that the PI signaling system is altered in the platelets of depressed and schizophrenic subjects. Kaiya et al. [17] showed an increase in DG in the platelets of schizophrenic subjects and proposed that this increase may cause a decrease in $Ins(1,4,5)P_3/Ca^{2+}$ function. Also Mikuni et al. [18] reported an increase in 5HT-induced accumulation of inositol phosphate-1 (IP_1) in the platelets of depressed patients compared to control subjects. We also observed that thrombin-stimulated IP_1 receptors formation was significantly greater in depressed

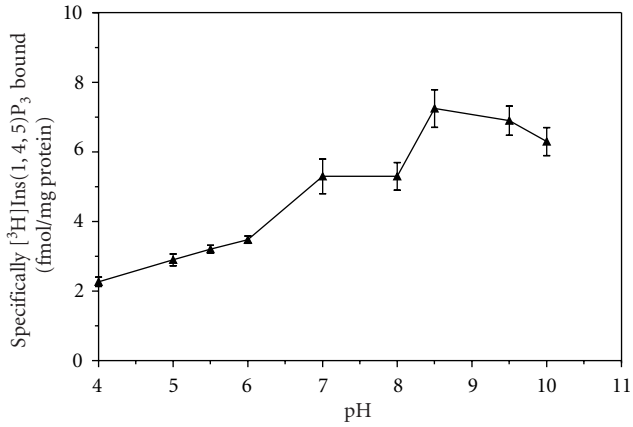

FIGURE 1: The effect of pH on $[^3H]Ins(1,4,5)P_3$ binding to human platelet membranes. Binding at different pH values was performed as described in Section 2. The reaction mixture contained 20 nM $[^3H]Ins(1,4,5)P_3$ and approximately 100 μg protein. Nonspecific binding was determined in the presence of 10 μM D-$Ins(1,4,5)P_3$. Incubations were carried out at 4°C for 10 minutes. Each point represents the mean value of two experiments performed in duplicate.

patients compared to control subjects [19]. Since the above-mentioned studies indicate abnormalities in the PI signaling system in depression, it is quite possible that these abnormalities may also be associated with alterations in $Ins(1,4,5)P_3$ receptors. Therefore, it is important to examine $Ins(1,4,5)P_3$ receptors in the platelets of these subjects. So far, the role of $Ins(1,4,5)P_3$ receptors in platelets of patients with mental disorders has not been studied. To eventually examine if $Ins(1,4,5)P_3$ receptors are altered in depressed subjects and patients with other mental disorders, we characterized $Ins(1,4,5)P_3$ receptors in crude platelet membranes obtained from normal human control subjects. Although $Ins(1,4,5)P_3$ receptors have been characterized in purified intracellular human platelet membranes rich in dense tubular systems [20], for clinical studies it is important to examine if $Ins(1,4,5)P_3$ receptors can be studied in crude membranes since it is not feasible to obtain large amounts of blood from patient populations.

Ca^{2+} and cAMP are two potent modulators in $Ins(1,4,5)P_3$ receptors in various tissues (5,21,22,23). Depending on its concentration, Ca^{2+} has been shown to alter $[^3H]Ins(1,4,5)P_3$ binding in various tissues (5,24,25). To further investigate if Ca^{2+} affects $[^3H]Ins(1,4,5)P_3$ binding, we studied the effects of various concentrations of $CaCl_2$ on $[^3H]Ins(1,4,5)P_3$ binding to crude platelet membranes.

cAMP is known to release Ca^{2+} from permeabilized platelets. cAMP also phosphorylates $Ins(1,4,5)P_3$ receptors via cAMP-dependent protein kinase A (PKA) [21]. However, whether cAMP affects $[^3H]Ins(1,4,5)P_3$ binding to platelet membranes, has not yet been studied. To determine if cAMP has any effect on $Ins(1,4,5)P_3$ receptors, we investigated the effect of forskolin, which is known to release endogenous cAMP, on $[^3H]Ins(1,4,5)P_3$ binding to crude platelet membranes.

Findings of the present study suggest that the pharmacological characteristics of $[^3H]Ins(1,4,5)P_3$ binding to crude platelet membranes are similar to that of $Ins(1,4,5)P_3$ receptors; and that both Ca^{2+} and cAMP modulate $[^3H]Ins(1,4,5)P_3$ binding in crude platelet membranes. With these properties, IP3 receptors can be successfully measured in platelet membranes that can be used to as diagnostic marker for major mental illnesses, including major depression, where abnormalities in PI signaling system have been reported.

2. Materials and Methods

2.1. Chemicals. D-myo-$[^3H]$inositol 1,4,5-trisphosphate (specific activity 21 Ci/mmol) was obtained from New England Nuclear (Boston, MA). D-myo-inositol 1,4,5-trisphosphate, L-myo-inositol 1,4,5-trisphosphate, D-myo-inositol 2,4,5-trisphosphate, L-α-glycerophosphoinositol 4,5-bisphosphate ($GPIP_2$), heparin sulfate, and forskolin were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade and were obtained from Sigma Chemical Co.

2.2. Preparation of Platelet Membranes. Blood (10 to 20 mL) was drawn from healthy normal human subjects into a tube containing 3.8% (w/v) sodium citrate. The blood was centrifuged immediately at 210 × g for 10 minutes at 4°C to obtain platelet-rich plasma (PRP), which was centrifuged at 4000 × g for 10 minutes at 4°C. The platelet pellet thus obtained was homogenized by polytron (at #7 setting) for 30 seconds in a homogenizing buffer containing 50 mM Tris-HCl, pH 7.7; 1 mM ethylene diamine N′, N′, N′, N′-tetraacetic acid (EDTA); and 2 mM 2-mercaptoethanol. The homogenate was centrifuged at 40,000 × g for 15 minutes at 4°C. The supernatant was discarded and the pellet was homogenized once again in the homogenizing buffer and centrifuged as described above. The resulting pellet was resuspended in a buffer containing 50 mM Tris-HCl, pH 8.5; 1 mM EDTA; and 1 mM 2-mercaptoethanol. This fraction was used for the $[^3H]Ins(1,4,5)P_3$ binding assay. To examine the effect of $CaCl_2$, the binding assay was performed in presence or absence of EDTA, as described below.

2.3. $[^3H]Ins(1,4,5)P_3$ Binding Assay. The binding of $[^3H]Ins(1,4,5)P_3$ to crude human platelet membranes was carried out in duplicate. The incubation medium contained incubation buffer (50 mM Tris-HCl, pH 8.5; 1 mM 2-mercaptoethanol; 1 mM EDTA), $[^3H]Ins(1,4,5)P_3$ (specific activity 21 Ci/mmol) ranging from 10 to 100 nM (six different concentrations), and 40 μL of platelet membrane suspension in a total volume of 100 μL. Nonspecific binding was determined in the presence of 10 μM $Ins(1,4,5)P_3$. The incubation was performed at 4°C for 10 minutes and rapidly terminated by the addition of 5 mL of cold buffer (50 mM Tris-HCl, pH 7.7; 1 mM EDTA; and 0.1% (w/v) bovine serum albumin) and filtration through Whatman GF/B filters. The filter-bound radioactivity was analyzed by a liquid scintillation counter. Specific binding was defined

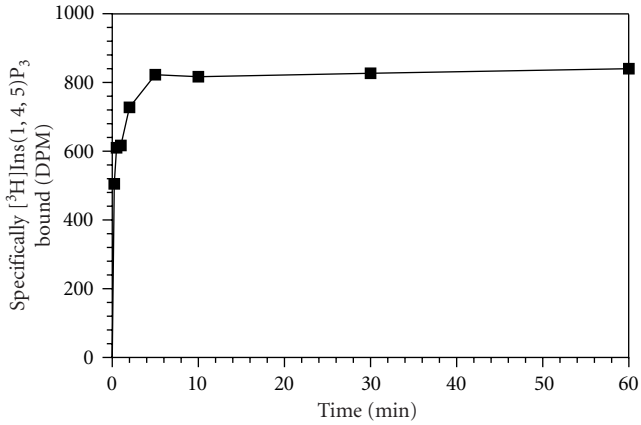

Figure 2: Time course of $[^3H]Ins(1,4,5)P_3$ binding to human platelet membranes. Platelet membranes (100 μg/protein) were incubated at 4°C for different time intervals in the presence of 20 nM $[^3H]Ins(1,4,5)P_3$. Nonspecific binding was estimated in the presence of 10 μM D-$Ins(1,4,5)P_3$. The incubations were rapidly terminated by vacuum filtration at different time points as shown in the figure. A single representative experiment is shown for duplicate determinations.

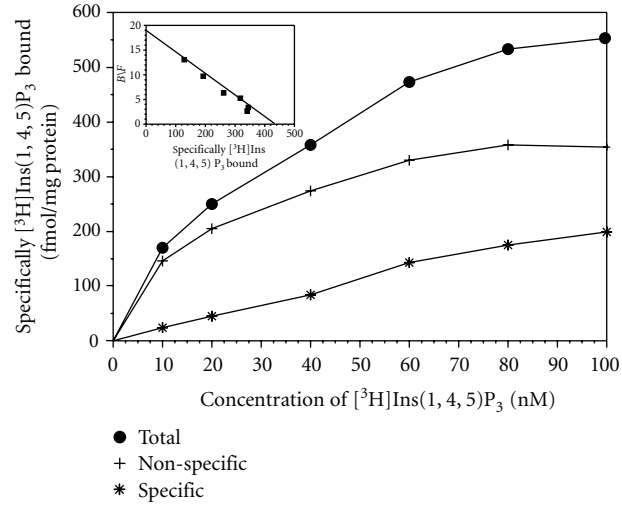

Figure 3: Saturation isotherm of $[^3H]Ins(1,4,5)P_3$ binding to human platelet membranes. Each point is the mean of duplicate determination. Binding assays were carried out as described in Section 2. Nonspecific binding was determined in the presence of 10 μM D-$Ins(1,4,5)P_3$. A Scatchard plot of $[^3H]Ins(1,4,5)P_3$ binding is shown in the inset. B = $[^3H]Ins(1,4,5)P_3$ specifically-bound (fmol/mg protein), B/F = the ratio of specifically-bound to free ligand in fmol of $Ins(1,4,5)P_3$ (fmol/mg protein × nM). For this particular experiment, binding indices are B_{max} = 427.56 fmol/mg protein K_d = 21.73 nM; and the correlation coefficient (r) = 0.98.

as the difference between the total binding and the binding observed in the presence of D-$Ins(1,4,5)P_3$. The maximum number of binding sites (B_{max}) and the apparent dissociation constant (K_d) were computed by Scatchard analysis using the EBDA program [22]. Protein was determined by the method of Lowry et al. [23]. IC_{50} (concentration of agents necessary to inhibit half of the specific $Ins(1,4,5)P_3$ binding) values of different agents (D-$Ins(1,4,5)P_3$; D-$Ins(2,4,5)P_3$; L-$Ins(1,4,5)P_3$; $GPIP_2$; and heparin) for the inhibition of specific binding were determined by log probit analysis.

To examine the pH-dependence of $[^3H]Ins(1,4,5)P_3$ binding and time course for specific binding of $[^3H]Ins(1,4,5)P_3$, 20 nM of $[^3H]Ins(1,4,5)P_3$ was used.

2.4. Determination of the Effect of $CaCl_2$ on $[^3H]Ins(1,4,5)P_3$ Binding. The effects of $CaCl_2$ were studied in the presence and in the absence of EDTA. Platelet membranes (100 μg protein) were incubated with $CaCl_2$ (0.5 to 30 mM) in a buffer containing 50 mM Tris-HCl, pH 8.5; 1 mM 2 mercaptoethanol; and 20 mM $[^3H]Ins(1,4,5)P_3$. EDTA (1 mM) was added to the incubation medium in which the effect of $CaCl_2$ was studied in the presence of EDTA. The incubation was carried out at 4°C for 10 minutes.

2.5. Determination of the Effect of Forskolin on $[^3H]Ins(1,4,5)P_3$ Binding. Platelet protein samples (100 μg) were incubated with forskolin (10^{-4} to 10^{-6} M) in a buffer containing 50 mM Tris-HCl, pH 8.5; 1 mM EDTA; and 1 mM 2-mercaptoethanol. The incubation was carried out at 4°C for 10 minutes.

3. Results

3.1. pH-Dependence of $[^3H]Ins(1,4,5)P_3$ Binding to Platelet Membranes. The results presented in Figure 1 show that $[^3H]Ins(1,4,5)P_3$ binding to crude platelet membranes is pH dependent. We measured specific binding of $[^3H]Ins(1,4,5)P_3$ between pH 4 and 10. Specific binding of $[^3H]Ins(1,4,5)P_3$ was relatively low at acidic pH (pH 4.0 to 6.0), and increased by 60% as the pH approached 7.0. Specific binding was stable from pH 7.0 to 8.0, but it increased further between pH 8.0 to 8.5, and thereafter declined.

3.2. Time Course for Specific Binding of $[^3H]Ins(1,4,5)P_3$ to Platelet Membranes. We determined the time course for $[^3H]Ins(1,4,5)P_3$ binding to crude platelet membranes from 15 seconds up to 60 minutes. As shown in Figure 2, the binding of $[^3H]Ins(1,4,5)P_3$ to $Ins(1,4,5)P_3$ receptors was very rapid. At 15 seconds the specific binding was very low but it reached equilibrium within 5 minutes. After that, specific binding remained constant up to 60 minutes.

3.3. Saturation Isotherm of $[^3H]Ins(1,4,5)P_3$ Binding to Platelet Membranes. The maximum number of binding sites (B_{max}) and the apparent dissociation constant (K_d) in crude platelet membranes were determined by using different concentrations of $[^3H]Ins(1,4,5)P_3$. Nonspecific binding was determined in the presence of 10 μM D-$Ins(1,4,5)P_3$. Initially, we performed the experiments using 0.1 to 100 nM $[^3H]Ins(1,4,5)P_3$. We observed that at lower concentrations of $[^3H]Ins(1,4,5)P_3$ (0.1 to 10 nM), the displacement was too low to draw the Scatchard plot. A concentration range of 10 to 100 nM $[^3H]Ins(1,4,5)P_3$, however, showed a specific binding of 80% to 50% depending upon the concentration

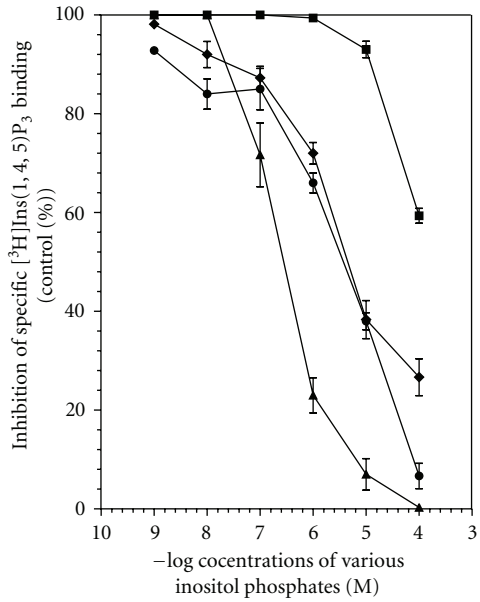

Figure 4: Displacement curve for the inhibition of $[^3H]Ins(1,4,5)P_3$ binding to human platelet membranes by inositol phosphates. Platelet membranes were incubated with 20 nM $[^3H]Ins(1,4,5)P_3$ in the presence of increasing concentrations of D-$Ins(1,4,5)P_3$ (▲), D-$Ins(2,4,5)P_3$ (•), $GPIP_2$ (♦), and L-$Ins(1,4,5)P_3$ (■) at 4°C for 10 minutes. The data are the mean ± S.E.M. of three independent experiments, each run in duplicate. The IC50 values were calculated using the displacement curve (Table 1).

Table 1: Apparent maximum binding sites (B_{max}) and dissociation constants (K_d) of $[^3H]Ins(1,4,5)P_3$ binding to human platelet membranes. Saturation analysis of $Ins(1,4,5)P_3$ binding sites was carried out using different concentrations of $[^3H]Ins(1,4,5)P_3$ as described in Section 2. Nonspecific binding was determined in the presence of 10 μM D-$Ins(1,4,5)P_3$. Each value is the mean ± S.E.M for five independent experiments performed in duplicate.

B_{max}	K_d
(fmol/mg protein)	(nM)
427.77 + 56.67	22.09 + 2.34

Table 2: Displacement of $[^3H]Ins(1,4,5)P_3$ binding by inositol phosphates and heparin in human platelet membranes. Platelet membranes were prepared as described in Section 2. The assay medium (100 μL) contained 20 nM $[^3H]Ins(1,4,5)P_3$ with various concentrations of competitive substances and approximately 100 μg of protein. Incubations were carried out at 4°C for 10 minutes. IC_{50} values were calculated by using log probit analysis. The values are the mean ± S.E.M. of three different experiments.

Compound	IC_{50}
—	(μM or *μg/mL)
D-$Ins(1,4,5)P_3$	0.3 ± 0.5
D-$Ins(2,4,5)P_3$	1.90 ± 0.68
$GPIP_3$	4.97 ± 1.83
L-$Ins(1,4,5)P_3$	354.0 ± 37.0
Heparin	36.79 ± 6.0l*

of $[^3H]Ins(1,4,5)P_3$. Figure 3 represents a typical saturation isotherm and a Scatchard plot (inset) of $[^3H]Ins(1,4,5)P_3$ binding to platelet membranes. Specific binding is saturable between 80 to 100 nM $[^3H]Ins(1,4,5)P_3$. Nonspecific binding is nonsaturable and linear with a concentration of 10 to 100 mM $[^3H]Ins(1,4,5)P_3$. The Scatchard plot indicates a single class of binding site. The means of B_{max} and K_d of five independent experiments performed in duplicate were found to be 427.77 ± 56.67 fmol/mg proteins and 22.09 ± 2.34 nM, respectively.

3.4. Specificity of $[^3H]Ins(1,4,5)P_3$ Binding. The pharmacological characterization of $Ins(1,4,5)P_3$ receptors was carried out using different agents known to inhibit $[^3H]Ins(1,4,5)P_3$ binding. A displacement curve of $[^3H]Ins(1,4,5)P_3$ binding with different concentrations of inositol phosphates is shown in Figure 4. Of the various inositol phosphates, D-$Ins(1,4,5)P_3$ was found to be the most potent inhibitor of $[^3H]Ins(1,4,5)P_3$ binding, with an IC_{50} value of 0.3 μM. Next in order were D-$Ins(2,4,5)P_3$ (IC_{50} = 1.9 μM), $GPIP_2$ (IC_{50} = 4.97 μM), and L-$Ins(1,4,5)P_3$ (IC_{50} = 354 μM). Heparin acts as an antagonist on the $Ins(1,4,5)P_3$ receptor and inhibited $[^3H]Ins(1,4,5)P_3$ binding in a concentration-dependent manner (5 to 500 μg/mL), with an IC_{50} value of 36.79 ± 6.0l μg/mL (Table 2 and Figure 5).

3.5. Effects of $CaCl_2$ on $[^3H]Ins(1,4,5)P_3$ Binding to Platelet Membranes. Ca^{2+} has been shown to be a potent modulator of $[^3H]Ins(1,4,5)P_3$ receptors. In the present investigation, we determined the effects of $CaCl_2$ on $[^3H]Ins(1,4,5)P_3$ binding to crude platelet membranes in the presence and in the absence of 1 mM EDTA. In the presence of EDTA, $CaCl_2$ inhibited $[^3H]Ins(1,4,5)P_3$ binding in a linear fashion depending upon its concentration. At a lower concentration (0.5 mM) the degree of inhibition was maximum (68%), while at a higher concentration (15 mM) the inhibition was very low (5%). At concentrations above 15 mM, however, $CaCl_2$ stimulated $[^3H]Ins(1,4,5)P_3$ binding, and at 30 mM $CaCl_2$, a four- to fivefold increase in $[^3H]Ins(1,4,5)P_3$ binding was observed (Figure 6). In another set of experiments, we observed the effects of $CaCl_2$ in the absence of EDTA. The results, shown in Figure 6(b), demonstrate that $CaCl_2$ increased $[^3H]Ins(1,4,5)P_3$ binding to platelet membranes between 24% to 64% depending on the concentration (2 to 15 mM). At a $CaCl_2$ concentration of 30 mM, the stimulation of $[^3H]Ins(1,4,5)P_3$ binding to crude platelet membranes was nearly the same (fourfold) as that observed in the presence of 1 mM EDTA.

3.6. Effects of In-Vitro Addition of Forskolin on $[^3H]Ins(1,4,5)P_3$ Binding in Platelet Membranes. Since cAMP has been shown to inhibit Ca^{2+} release from permealized platelets, and there is indirect evidence which shows that cAMP causes the phosphorylation of $Ins(1,4,5)P_3$ receptors by stimulating PKA, we studied the effects of forskolin on $[^3H]Ins(1,4,5)P_3$ binding to platelet membranes. Forskolin is a potent stimulator of adenylyl cyclase in platelets and thus increases cAMP levels of hydrolyzing adenosine trisphosphate (ATP).

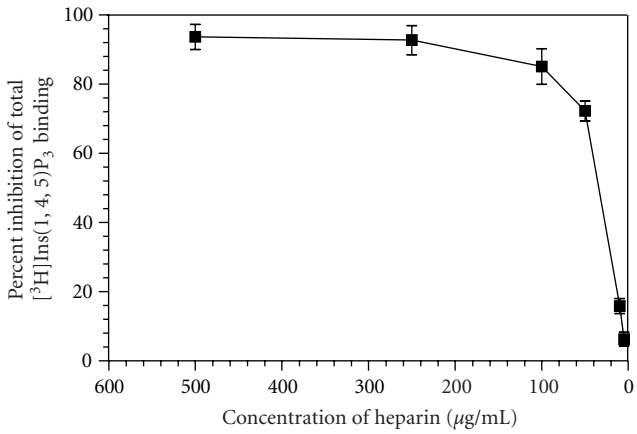

FIGURE 5: Inhibition of $[^3H]Ins(1,4,5)P_3$ binding to human platelet membranes by heparin. Experimental conditions are similar to those described in Figure 4. The points represent the mean ± S.E.M. of three independent experiments, each run in duplicate.

We added different concentrations of forskolin in vitro, and the results, given in Figure 7, show that forskolin inhibited $[^3H]Ins(1,4,5)P_3$ binding in a concentration-dependent manner. At a concentration of 10^{-4} M, inhibition was about 80%, whereas at lower concentrations (10^{-5} to 10^{-6} M), forskolin inhibited $Ins(1,4,5)P_3$ binding by 20% to 30%.

4. Discussion

The pharmacological properties of $Ins(1,4,5)P_3$ receptors have been characterized in several tissues including the brain [5, 8]. In an earlier study, Varney et al. [24] observed a single $[^3H]Ins(1,4,5)P_3$ binding site in crude platelet membranes; however, they did not fully characterize $Ins(1,4,5)P_3$ receptors in this fraction of platelets. To investigate if $Ins(1,4,5)P_3$ receptors in crude platelet membranes possess similar pharmacological properties as observed in other tissues, we characterized $[^3H]Ins(1,4,5)P_3$ binding sites in crude platelet membranes obtained from normal human control subjects. We observed a single, saturable binding site of $[^3H]Ins(1,4,5)P_3$ in crude platelet membranes, with a binding capacity of 427.77 fmol/mg protein and an affinity of 22.09 nM. The binding of $[^3H]Ins(1,4,5)P_3$ was pH- and time dependent. The optimum pH was found to be 8.5, with a steep change occurring in the pH range of 4 to 9. Time-course experiments revealed that maximum binding occurred at 2 minutes and remained stable up to 60 minutes. We determined IC_{50} values of different inositol phosphates in crude platelets membranes. D-$Ins(1,4,5)P_3$ was found to be the most potent, with an IC_{50} value of 0.3 μM. Heparin is a competitive antagonist of the $Ins(1,4,5)P_3$ receptor [5]. In the present study, heparin inhibited $[^3H]Ins(1,4,5)P_3$ binding in a concentration-dependent manner, with an IC_{50} value of 36.79 μg/mL. These pharmacological properties of $Ins(1,4,5)P_3$ receptors in crude platelet membranes were found to be similar to those reported in other tissues [5, 8]. Earlier, Hwang [20] characterized $[^3H]Ins(1,4,5)P_3$ receptors in purified human platelet membranes rich in dense tubular systems. In this fraction of platelet membranes, Hwang reported two binding sites of $[^3H]Ins(1,4,5)P_3$, one with low affinity, and another with high affinity; however in our study, we observed only one binding site in crude platelet membranes, which is very similar to that reported by Varney et al. [24]. One binding site has also been reported in other tissues [6, 10, 25]. It is quite possible that the explanation of the single binding site observed by us and Varney et al. in crude platelet membranes and the two binding sites observed by Hwang in purified platelet membranes may be the differences in the preparation of platelet membranes. The pH- and the time-course profiles observed in the present study are similar to those reported in other tissues, including platelets [5, 7, 9, 20].

In continuation of our study, we further investigated the effects of agents that are known to modulate $Ins(1,4,5)P_3$ receptors. One such agent is Ca^{2+}, which has been shown to affect $[^3H]Ins(1,4,5)P_3$ binding in most tissues [5, 25, 26]. In the present investigation, we studied the effects of various concentrations of $CaCl_2$ on $[^3H]Ins(1,4,5)P_3$ binding to crude platelet membranes. Since EDTA is known to chelate Ca^{2+} and our incubation medium contained 1 mM EDTA, we studied the effects of $CaCl_2$ in the presence and in the absence of EDTA. We observed that $CaCl_2$ stimulated $[^3H]Ins(1,4,5)P_3$ binding in a concentration-dependent manner (2 to 15 mM), and at a concentration of 30 mM, the stimulation was four- to fivefold. In the presence of EDTA, however, $CaCl_2$ inhibited $[^3H]Ins(1,4,5)P_3$ binding. We observed maximum inhibition at 0.5 mM, and the degree of inhibition decreased as the concentration of $CaCl_2$ increased (0.5 to 15 mM); and at a concentration of 30 mM, $CaCl_2$ stimulated $[^3H]Ins(1,4,5)P_3$ binding, the degree of stimulation being similar to that observed in the absence of EDTA. These results suggest a biphasic response of $CaCl_2$ on $[^3H]Ins(1,4,5)P_3$ binding to platelet membranes.

The mechanism by which Ca^{2+} inhibits $[^3H]Ins(1,4,5)P_3$ binding in platelets is presently unclear. Delfert et al. [27] reported an inhibitory effect in the release of Ca^{2+} from the endoplasmic reticulum in the presence of free Ca^{2+}. It is possible that Ca^{2+} itself regulates the further release of Ca^{2+} by inhibiting $Ins(1,4,5)P_3$ binding. Danoff et al. [28] hypothesized that the inhibitory effect of Ca^{2+} is mediated by a protein called calmedin in the cerebral membranes of rats; while Mignery et al. [29] suggested that Ca^{2+}-induced inhibition is mediated by Ca^{2+}-activated PLC, which produces additional $Ins(1,4,5)P_3$, with an apparent decrease in $[^3H]Ins(1,4,5)P_3$ binding. Whether this mechanism exists in platelets is not known at the present time.

Our studies also indicate that EDTA markedly alters the effect of $CaCl_2$ on $[^3H]Ins(1,4,5)P_3$ binding. There is a possibility that 1 mM EDTA chelated most of the Ca^{2+}, leaving a micromolar concentration of Ca^{2+}, which was able to inhibit $[^3H]Ins(1,4,5)P_3$ binding, since it has been shown that inhibition of $[^3H]Ins(1,4,5)P_3$ binding occurs at micromolar concentrations of Ca^{2+} [30]. However, when we increased the concentration of $CaCl_2$ to 30 mM, there was not enough EDTA present in the medium to chelate Ca^{2+}, and the concentration of Ca^{2+} present in the medium was high enough to stimulate $[^3H]Ins(1,4,5)P_3$

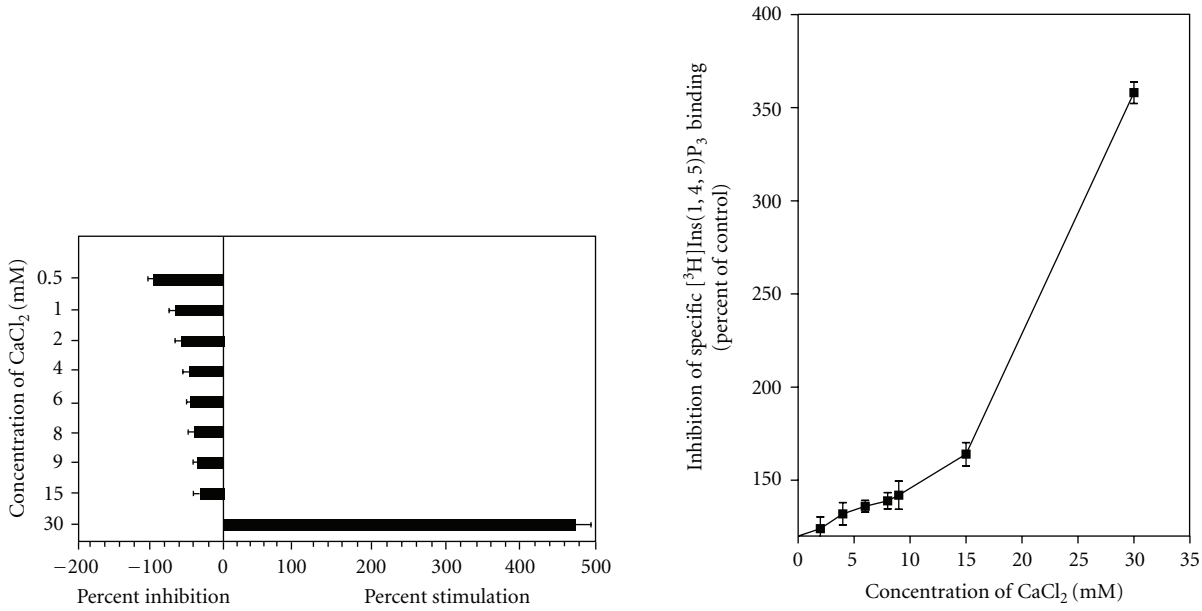

FIGURE 6: Effects of $CaCl_2$ on $[^3H]Ins(1,4,5)P_3$ binding to human platelet membranes in the presence (a) and absence (b) of EDTA. Platelet membranes were incubated with increasing concentrations of $CaCl_2$ in the presence of 20 nM $[^3H]Ins(1,4,5)P_3$ in a buffer containing 50 mM Tris-HCl, pH 8.4; 1 mM EDTA; and 1 mM 2-mercaptoethanol at 4°C for 10 minutes. The data represent the inhibition or stimulation of $[^3H]Ins(1,4,5)P_3$ binding to platelet membranes. The points represent the mean ± S.E.M. of three independent experiments, each run in duplicate.

binding. In the absence of EDTA, however, $CaCl_2$ stimulated $[^3H]Ins(1,4,5)P_3$ binding. The mechanism by which $CaCl_2$ potentiated the binding is not known at this present time and needs further study. Nonetheless, this study suggests that $CaCl_2$ modulates $[^3H]Ins(1,4,5)P_3$ binding in platelets.

Another important modulator in $Ins(1,4,5)P_3$ receptors is cAMP, which has been shown to release Ca^{2+} from platelet membranes and is involved in phosphorylation of $[^3H]Ins(1,4,5)P_3$ receptors [31, 32]. To investigate the effects of cAMP on $[^3H]Ins(1,4,5)P_3$ binding, we added forskolin in vitro to the assay medium. Forskolin is known to act directly on adenylyl cyclase, thereby generating endogenous cAMP. We found that forskolin inhibited $[^3H]Ins(1,4,5)P_3$ binding in platelets in a concentration-dependent manner. The mechanism by which cAMP inhibits $[^3H]Ins(1,4,5)P_3$ binding is not clear at this present time. It is quite possible that the inhibition observed in $[^3H]Ins(1,4,5)P_3$ binding to platelet membranes by forskolin might be due to the phosphorylation of $Ins(1,4,5)P_3$ receptors by cAMP-dependent PKA [31, 32]. The possibility that forskolin acts directly on $[^3H]Ins(1,4,5)P_3$ binding cannot be ruled out, however.

In summary, our study shows a single, saturable binding site for $[^3H]Ins(1,4,5)P_3$ in crude platelet membranes, which is time dependent and modulated by pH, inositol phosphates, heparin, Ca^{2+}, and cAMP. Although there is a minor difference between the results obtained in crude platelet membranes in this study as compared to the results in purified platelet membranes, the pharmacological

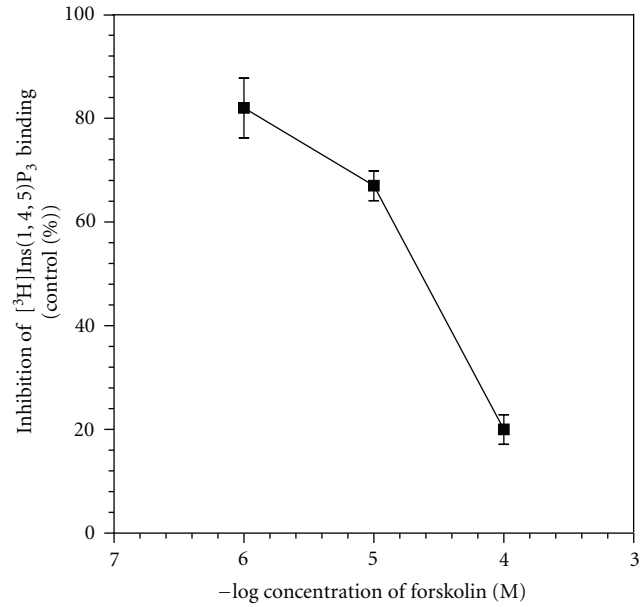

FIGURE 7: Inhibitory effect of forskolin on $[^3H]Ins(1,4,5)P_3$ binding to human platelet membranes. The experiments were performed in an assay medium (50 mM Tris HC1, pH 8.4; 1 mM EDTA; 1 mM 2-mercaptoethanol) containing 30 mM $CaCl_2$ 20 nM $[^3H]Ins(1,4,5)P_3$, and increasing concentrations of forskolin at 4°C for 10 minutes. The data are the mean ± S.E.M. of three independent experiments, each carried out in duplicate.

characteristics of [^{3}H]Ins(1,4,5)P_3 binding to crude platelet membranes are similar to the pharmacological properties of Ins(1,4,5)P_3 receptors in other tissues, including platelets. These results are important, especially considering that due to the difficulty of obtaining large-enough samples of blood from patients, preparation of purified platelet membranes is not feasible for clinical research. Since the preparation of crude platelet membranes is convenient and requires a smaller amount of blood, this procedure can be utilized to study the role of Ins(1,4,5)P_3 receptors in depression and other mental disorders, such as schizophrenia or bipolar disorders. Even, within a specific diagnosis, measuring IP3 receptors may be helpful in distinguishing subtypes of mental illness. For example, it will be interesting to examine whether IP3 receptors are altered in a subset of depressed patients. In this regard, the prime example is protein kinase A, which has been shown to be altered in a subtype of depressed patients, that is, melancholic depressed patients or patients who committed suicide [33, 34]. Thus, measuring IP3 receptors in blood cells may lead to the development of novel interventions that could target specific points of vulnerability.

Abbreviations

B_{max}	Maximum number of binding sites
cAMP	Cyclic adenosine monophosphate
EDTA	Ethylene diamine N', N', N', N'-tetraacetic acid
GPIP2	L-α-glycerophosphoinositol 4,5-bisphosphate
K_d	Apparent dissociation constant
Ins(1,4,5)P_3	Inositol (1,4,5) triphosphate
PKA	Protein kinase A
PI	Phosphatidylinositol
PKC	Protein kinase C

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