Volume 257, number 2, 427–430 FEB 07806 November 1989

Activation of protein kinase C alters the interaction of α_2-adrenoceptors and the inhibitory GTP-binding protein (G_i) in human platelets

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Received 18 September 1989

The effect of 12-tetradecanoyl phorbol 13-acetate (TPA) on the hormonal modulation of adenylate cyclase was studied. The effect of epinephrine (α_2-adrenergic action) was markedly diminished in membranes from TPA-treated platelets as compared to the controls. Interestingly, the inhibitory effect of guanylyl imido diphosphate (Gpp(NH)p) was not altered. Neither the number of α_2-adrenoceptors nor their affinity for [^{3}H]yohimbine were affected by the treatment with TPA. In control platelets, 77% of the receptors were in a high-affinity state for epinephrine and 22% in a low-affinity state; Gpp(NH)p shifted the receptor affinity towards the low-affinity conformation. In membranes from TPA-treated platelets, the receptors were in the low-affinity state and no further decrease in affinity was induced by Gpp(NH)p. Our data suggest that activation of protein kinase C in platelets blocks the hormonal inhibition of adenylate cyclase by interfering with the receptor-G_i interaction.

Protein kinase C; Enzyme activation; Receptor-protein interaction; Adrenoceptor

1. INTRODUCTION

Protein kinase C participates in the intracellular propagation of signals that act through the calcium-phosphoinositide transduction system [1]. However, its importance goes much beyond this, protein kinase C provides positive forward as well as negative feedback controls over various steps of its own and other signalling pathways [2].

The adenylate cyclase complex (receptors, G-proteins and the catalytic subunit) seems to be a target of protein kinase C and thus, one of the sites through which major interaction between these two signalling pathways takes place [2]. It has been observed that activation of protein kinase C modulates the activation of adenylate cyclase induced by hormones and neurotransmitters [3–5] and impairment of the hormone-sensitive inhibitory pathway of adenylate cyclase by protein kinase C has been reported [6–9].

Treatment of human platelets with 12-tetradecanoyl phorbol 13-acetate (TPA) largely impairs the GTP-dependent hormone-sensitive inhibitory pathway to adenylate cyclase which involves the inhibitory GTP-binding protein, G_i [6–9]. Protein kinase C phosphorylates G_i [7] and suppresses its function in hormonal inhibition of adenylate cyclase [6–9].

Guanine nucleotide-binding proteins seem to exert two basic functions: first, to regulate the activity of membrane effectors (i.e. to activate or inhibit enzymes, such as adenylate cyclase phospholipase A_2 or phospholipase C and ionic channels) [10,11] and second, to modulate the affinity state of hormone receptors [12].

Using human platelets we examined the effect of TPA on these two functions of G_i; our results suggest that activation of protein kinase C uncouples G_i from the α_2-adrenoceptors without altering the interaction of G_i with adenylate cyclase.

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2. MATERIALS AND METHODS

Blood was obtained from healthy men and women who had taken no medication during the previous 2 weeks. Platelet-rich plasma was obtained by centrifugation. In some experiments the platelet-rich plasma was incubated with 1 mM aspirin for 30 min at 37°C to inhibit cyclooxygenase [13]; this treatment did not alter the results obtained. After a preequilibration period of 5 min at 37°C, the platelets were challenged with 1 μM TPA, or vehicle for 1 min; the platelets were centrifuged and homogenized. A crude membrane preparation was obtained as described by Hoffman et al. [14].

Adenylate cyclase activity was assayed in a mixture containing 25 mM Tris (pH 7.5), 5 mM $MgCl_2$, 0.1 mM ATP (containing [α-^{32}P]ATP 500000 cpm per tube), 5 mM theophylline, 2.5 mg/ml phosphocreatine and 1 mg/ml creatine kinase; the reaction was started by the addition of membrane protein (50 μg) and it was carried out for 20 min at 30°C in a total volume of 0.1 ml. Cyclic AMP was isolated by the method of Salomon et al. [15].

[^{3}H]Yohimbine binding studies were performed as described by Hoffman et al. [14]. The binding competition experiments were analyzed by computer modelling techniques [15–17].

3. RESULTS

Basal adenylate cyclase activity was similar in membranes from control and TPA-treated platelets (26 ± 6

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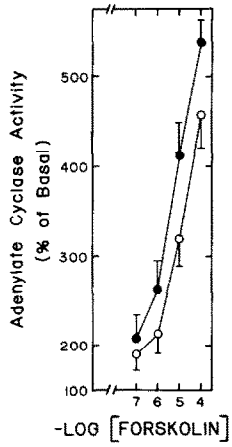

Fig.1. Effect of TPA on the stimulation of adenylate cyclase activity by forskolin. Membranes from control (open circles) or TPA-treated (closed circles) platelets were incubated with different concentrations of forskolin. Results are expressed as % of basal activities. Plotted are the means and vertical lines represent the SE of 6 experiments in triplicate using different membrane preparations.

and 25 ± 5 pmol/min per mg protein for membranes for control and TPA-treated platelets, respectively (means ± SE, n = 10)). Forskolin stimulated adenylate cyclase activity in a dose-dependent fashion in membranes from control and TPA-treated platelets (fig.1). However, the activation induced by the diterpene was slightly greater in membranes from TPA-treated platelets than in the controls (fig.1). Epinephrine induced a dose-dependent inhibition of forskolin-stimulated adenylate cyclase activity (fig.2); the effect of epinephrine was blocked by 10 μM yohimbine indicating the involvement of α_2-adrenoceptors (not shown). In agreement with the data of Jakobs et al. [6], we observed that the effect of epinephrine was markedly reduced in membranes from TPA-treated platelets as compared to the controls, i.e. 100 μM epinephrine induced a 35–40% inhibition of adenylate cyclase activity in control membranes whereas in membranes from TPA-treated platelets the same concentration of epinephrine induced only a 5–10% inhibition (fig.2). The effect of the treatment with TPA was not exclusive for epinephrine. The action of other agents that inhibit adenylate cyclase through their own receptors such as thrombin and platelet activating factor (PAF) was also similarly diminished (data not shown). Interestingly, the inhibitory action of the hydrolysis resistant analogue of GTP, Gpp(NH)p, was identical in membranes from control and TPA-treated platelets (fig.2).

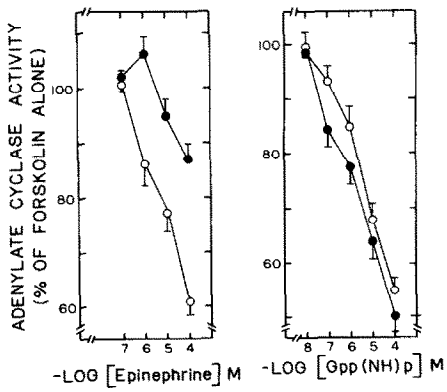

Fig.2. Effects of epinephrine or Gpp(NH)p on forskolin-stimulated adenylate cyclase activity. Membranes from control (open circles) or TPA-treated (closed circles) platelets were incubated with 100 μM forskolin and either 10 μM GTP and different concentrations of epinephrine (left panel) or different concentrations of Gpp(NH)p (right panel). Results are expressed as % of the adenylate cyclase activity observed in the presence of forskolin alone. Plotted are the means and vertical lines represent the SE of 10 experiments in triplicate using different membrane preparations.

Table 1

Effect of TPA on [^{3}H]yohimbine binding

Treatment	B_{max} (fmol/mg per protein)	K_d (nM)
Control	118 ± 10	3.2 ± 0.3
TPA	140 ± 19	3.5 ± 0.3

Membranes from control or TPA-treated platelets were incubated with [^{3}H]yohimbine as described in section 2. Results are the means ± SE from 3 separate experiments in triplicate

We next examined the effect of TPA treatment on the α_2-adrenoceptor number and affinity using [^{3}H]yohimbine. Scatchard plots of the binding data were linear, consistent with a single type of receptor; no significant difference in B_{max} or K_d was observed between control membranes and those from TPA-treated platelets (table 1). The data indicate that neither the number of sites nor their affinity for the antagonist is altered by the treatment with TPA.

In membranes from control platelets, the displacement of [^{3}H]yohimbine binding by epinephrine was dose-dependent (fig.3) and gave a shallow curve with a

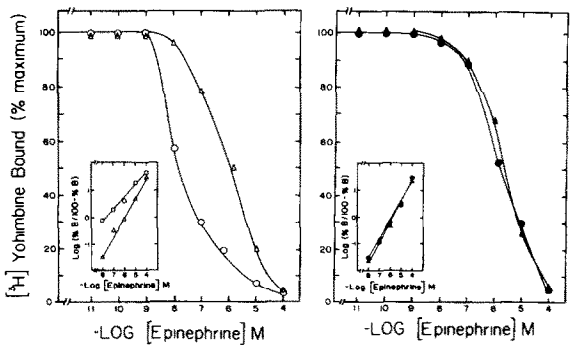

Fig.3. Displacement by epinephrine of specific [^{3}H]yohimbine binding. Membranes from control (open symbols) or TPA-treated platelets (solid symbols) were incubated with 7 nM [^{3}H]yohimbine and different concentrations of (−)epinephrine in the absence (circles) or presence (triangles) of 100 μM Gpp(NH)p. Plotted is a representative experiment of 6 replicates with different membrane preparations. The insets show the Hill analysis of the competition studies.

Table 2

Parameters derived from the computer modelling of competition curves of epinephrine with [^{3}H]yohimbine

Treatment	Agents	[^{3}H]Yohimbine				
		K_1 (nM)	%	K_2 (nM)	%	Hill coefficient
Control	epinephrine[a]	20 ± 20	77 ± 5	3000 ± 700	22 ± 6	0.45 ± 0.03
	epinephrine + Gpp(NH)p	–	–	2000 ± 100	100	0.72 ± 0.02[b]
TPA	epinephrine	–	–	1700 ± 100	100	0.74 ± 0.04
	epinephrine + Gpp(NH)p	–	–	1800 ± 200	100	0.72 ± 0.04

[a] Two-state fit significantly better than one-state fit ($P < 0.05$)

[b] $P < 0.001$ as compared to epinephrine alone, control

Results are the means ± SE of 6 experiments in triplicate using different membrane preparations

Hill coefficient of 0.45, which suggests the presence of heterogeneous binding sites. Addition of 0.1 mM Gpp(NH)p induced an approximately 30-fold shift to the right in the displacement curve and an increase in the Hill slope to 0.72 (fig.3). This Hill slope suggests that some heterogeneity may still persist in the binding sites; similar results have been observed by other authors [18] but the reason is unknown. Computer modelling of the data indicated in the absence of Gpp(NH)p the presence of two classes of binding sites with high (K_1) and low (K_2) affinities for epinephrine (table 2); most of the receptors were in the high-affinity state for agonists. The competition curve in the presence of Gpp(NH)p gave a binding isotherm consistent with a single class of binding sites whose affinity for epinephrine was similar to the K_2 observed in the absence of Gpp(NH)p (table 2).

The displacement of [^{3}H]yohimbine binding by epinephrine in membranes from TPA-treated platelets showed two important differences with the controls (fig.3): first, the displacement curve in the absence of Gpp(NH)p was steeper (Hill, 0.74) and shifted to the right; second, no further effect of Gpp(NH)p was observed (fig.3). Computer modelling indicated that in these membranes a single type of sites was detected regardless of the presence or absence of Gpp(NH)p (table 2).

4. DISCUSSION

Our present data confirm and extend those of Jakobs and co-workers [6–9] and indicate that activation of protein kinase C leads to an impairment of the hormone-sensitive inhibitory branch of adenylate cyclase. The alteration of the inhibitory branch of adenylate cyclase was observed for three agents (epinephrine, PAF, thrombin) acting through independent receptors. These data are consistent with the idea that the coupling between these receptors and the catalytic subunit of adenylate cyclase is affected by protein kinase C; such coupling is mediated via the inhibitory guanine nucleotide-binding regulatory protein, G_i.

Interestingly, the inhibitory effect of Gpp(NH)p on forskolin-stimulated adenylate cyclase activity was not altered in membranes from TPA-treated platelets, which suggest that the mechanism(s) through which G_i inhibits adenylate cyclase are not altered by protein kinase C. In other words, the data suggest that the alteration induced by protein kinase C on G_i does not affect its interaction with the catalytic subunit of adenylate cyclase but rather that it is the receptor-G_i interaction that is affected. Direct evidence for an altered receptor-G_i interaction was obtained in the binding studies. However, the possibility of an additional defect in the G_i-adenylate cyclase interaction cannot be completely ruled out. Two reasons exist for such reserve: (i) firstly, our understanding of the mechanism(s) through which adenylate cyclase is inhibited by G_i is incomplete [19–21] and (ii) secondly, it is not known if the mechanisms of activation of G_i by hydrolysis resistant analogues of GTP are identical to those of the natural nucleotide. Regarding this latter point, GTP has a biphasic effect on human platelet adenylate cyclase, increasing enzyme activity at submicromolar concentrations and inducing inhibition at higher concentrations [8]; TPA blocks the inhibitory phase of GTP action [8]. However, hydrolysis-resistant analogues of GTP, such as GTP[S] or Gpp(NH)p (this manuscript), inhibit adenylate cyclase similarly in membranes from control and TPA-treated platelets.

There is evidence that many receptors including α_2-adrenoceptors exist in two interconvertible affinity states for agonists (high- and low-affinity states). The conversion of the low-affinity state to the high-affinity state seems to involve the interaction with G-proteins; reconstitution of the high-affinity state for agonists of platelet α_2-adrenoceptors with exogenous G_i has already been reported [22]. Our binding data indicate that in membranes from TPA-treated platelets, α_2-adrenoceptors remain in the low-affinity state for agonists which suggests that the receptor-G_i interaction

is perturbed by pretreatment with TPA and that therefore the α_2-adrenoceptors are unable to form the high-affinity state for agonists. Remarkable similarities exist between the effects of TPA on platelets reported here and those of pertussis toxin on other cells.

Acknowledgements: The authors thank Ms Guadalupe Ramírez for typing the manuscript. The authors also thank the blood donors and Miss Lucía Yáñez for obtaining the blood samples. This research was supported in part by grants from CONACyT (ICEXCNA 060394), The Third World Academy of Sciences (TWAS RGBC 88-93) and Fundación Miguel Alemán.

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