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INDUCTION OF DESENSITIZATION BY PHORBOL ESTER TO β-ADRENERGIC AGONIST STIMULATION IN ADENYLATE CYCLASE SYSTEM OF RAT RETICULOCYTES

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Summary: Treatment of rat reticulocytes with a phorbol ester, tetradecanoyl phorbol acetate (TPA), resulted in the desensitization of adenylate cyclase to the β-adrenergic agonist stimulation depending on the dose and period of the TPA treatment. Treatment of the reticulocytes with TPA caused approximately 40 % reduction in the stimulation by β-adrenergic agonists of adenylate cyclase activity, whereas the treatment had little effect on the basal activity and the activation by fluoride and guanine nucleotide of the enzyme system. No change in the number of β-adrenergic receptors was observed after the TPA treatment. Treatment with 1-oleoyl-2-acetyl-glycerol (OAG), an activator of protein kinase C, also caused the desensitization of reticulocyte adenylate cyclase to isoproterenol. On the other hand, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), a potent inhibitor of protein kinase C, prevented the desensitization induced by TPA. These results suggest the involvement of protein kinase C in a process of desensitization of adenylate cyclase system to β-adrenergic agonists in rat reticulocytes.

Prolonged exposure of cells and tissues to stimulants, such as hormone, neurotransmitter, etc., attenuates their responsiveness to further stimulation. The phenomenon, called "desensitization" or "refractoriness", has most widely been studied for adenylate cyclase system in cell membrane. Mechanism for the desensitization has been examined mainly for amphibian and avian erythrocytes and cultured cells (1,2). Recently, it has been reported that phorbol esters, such as tetradecanoyl phorbol acetate (TPA), etc., induce the desensitization to β-adrenergic agonists in adenylate cyclase system of rat glioma C6 cells (3,4) and avian erythrocytes (5,6). Since several reports suggest that the high affinity binding site for phorbol ester corresponds to

Abbreviations: TPA, tetradecanoyl phorbol acetate; OAG, 1-oleoyl-2-acetyl-glycerol; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; GppNHp, guanylyl 5'-imidodiphosphate; Ns, guanine nucleotide-binding stimulatory regulatory protein in adenylate cyclase system; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate ; DHA, dihydroalprenolol.

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Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) (7-9), we have attempted to examine the involvement of protein kinase C in TPA-induced desensitization to β-adrenergic agonists in adenylate cyclase system of rat reticulocytes. In this paper, results will be presented that 1-oleoyl-2-acetylglycerol (OAG), a potent protein kinase C activator (10,11), also causes desensitization to β-adrenergic agonists as well as TPA in the adenylate cyclase system, and that 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), a potent inhibitor of protein kinase C (12), prevents the desensitization induced by TPA.

MATERIALS AND METHODS

Isoproterenol, ATP, OAG, and GppNHp were purchased from Sigma Chemical Co., [^{3}H]ATP and [^{3}H]dihydroalprenolol(DHA) from Amersham International Ltd., and GTP from Yamasa Co. All other agents were of reagent grade from standard commercial sources. H-7 and TPA were kind gifts from Prof. H. Hidaka, Mie University School of Medicine and Prof. T. Ando, Meiji College of Pharmacy, respectively.

Male rats of Wistar strain (150-200 g) were subcutaneously injected with 1 mg/kg/day of phenylhydrazine hydrochloride for 6 days. At the 8th day, the rats were killed by exsanguination from the carotid artery under ether anesthesia, and whole blood was collected into a tube containing saline added with 10 mM EDTA. The blood was washed 3 times by centrifugation at 500g for 10 min. Buffy coat was carefully discarded, and final pellet was suspended in 10 vols. of Krebs Ringer Hepes buffer (pH 7.4). Abundance of the reticulocytes in the blood was confirmed by a microscopic examination after Brilliant Cresyl Blue staining.

The reticulocytes were suspended in Krebs Ringer Hepes buffer (pH 7.4), and incubated in the presence of various concentrations of TPA at 30°C for up to 120 min. Then, the reticulocytes were sedimented by centrifugation, washed 3 times with the same buffer, and lysed hypotonically by addition of 10 vols. of 5 mM Tris-HCl/1 mM EDTA (pH 7.4). The lysate was centrifuged at 20,000g, and the pellet was suspended and washed 3 times with the same buffer as above. The final pellet was suspended in 50 mM Tris-HCl buffer (pH 7.4), and used as membrane preparation in the following experiments. In some cases, the reticulocytes were treated with 10 or 100 μM OAG at 30°C for 90 min, or with 1 μM TPA in the presence of various concentrations of H-7 at 30°C for 90 min in the similar manner.

Adenylate cyclase activity was measured according to the method of Salomon, et al. (13) with minor modifications (14). Protein was determined by the method of Lowry, et al. (15).

β-Adrenergic receptor number was determined by the method of Mukherjee, et al. (16) with the use of [^{3}H]DHA. To the membrane preparation suspended in 50 mM Tris-HCl and 15 mM $MgCl_2$ (pH 8.0), [^{3}H]DHA was added to a final concentration of 0-50 nM, and the mixture was incubated at 30°C for 10 min. At the end of the incubation, 5 vols. of chilled incubation buffer was added, and the mixture was rapidly centrifuged. The pellet was washed 3 times with the same buffer, and then solubilized with 10 % SDS. An aliquot of the solution was measured for radioactivity with a liquid scintillation spectrometer. Specific binding of [^{3}H]DHA was calculated by subtracting non-specific binding in the presence of 20 μM alprenolol from the total binding.

Table 1: Desensitization of adenylate cyclase to β-adrenergic agonists in TPA-treated reticulocytes

Addition to membrane	Adenylate cyclase activity (pmol/min/mg protein)		% of control
	non-treated	TPA-treated	
none	2.2 ± 0.2	2.2 ± 0.3	100
10 μM isoproterenol	40.0 ± 2.5	25.0 ± 2.1	62.5
10 μM epinephrine	37.5 ± 3.6	23.5 ± 2.2	62.7
10 μM norepinephrine	35.7 ± 1.1	22.3 ± 2.3	62.5
5 mM NaF-20 μM $AlCl_3$	26.4 ± 2.6	26.8 ± 2.9	101.5
10 μM GppNHp	24.3 ± 2.7	24.4 ± 3.0	100.4

Rat reticulocytes were incubated for 90 min in either the presence or absence of 1 μM TPA. Membranes were prepared from the reticulocytes, and assayed for adenylate cyclase activity in the presence of the indicated additives. Values represent mean ± S.D. (n=4).

RESULTS AND DISCUSSION

Rat reticulocytes were treated with 1 μM TPA for at 30°C for 90 min, and then adenylate cyclase activity in the reticulocyte membrane was measured in the presence or absence of various β-adrenergic agonists as shown in Table 1. The agonist-stimulated adenylate cyclase activity in the TPA-treated reticulocytes was about 60 % of that in the untreated control reticulocytes, suggesting that TPA induced desensitization to β-adrenergic agonists of the adenylate cyclase system in rat reticulocytes. The desensitization was dependent on both the dose of TPA and the period of the treatment (Fig. 1). Since the TPA

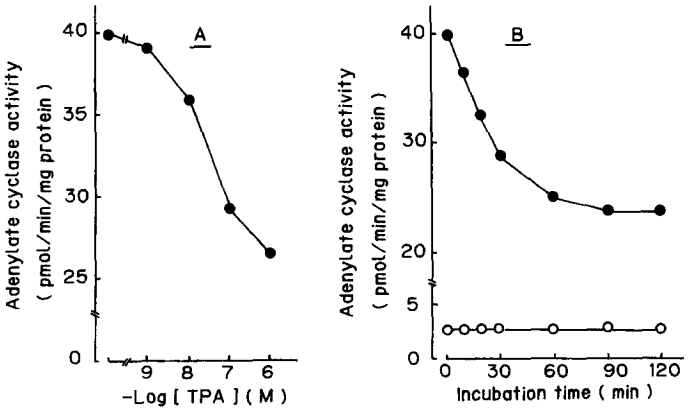

Fig. 1. Dependence of desensitization of adenylate cyclase system in rat reticulocytes to isoproterenol on the concentration of TPA and period of the treatment with TPA. Rat reticulocytes were incubated at 30°C with various concentrations of TPA for 90 min (Panel A) or with 1 μM TPA for various periods (Panel B), and membranes were prepared from the reticulocytes and assayed for adenylate cyclase activity. Panel A: The activity was measured in the presence of 10 μM isoproterenol. Panel B: The activity was measured in the presence (●) or absence (○) of 10 μM isoproterenol.

Table 2: Binding coefficients for β-adrenergic ligand in reticulocytes treated with TPA

	non-treated	TPA-treated
K_D for ^{3}H-DHA (nM)	5.0 ± 0.1	4.9 ± 0.1
B_{max} (fmol/mg protein)	100.4 ± 5.0	100.3 ± 2.0

Membranes were prepared from the reticulocytes, and assayed for ^{3}H-DHA binding as described in the text. Others are the same as in Fig. 1, except for n=3.

treatment had no effect on GppNHp or fluoride-stimulated adenylate cyclase activity, the desensitization seemed to be due to the modification of β-adrenergic receptors. But, no change was observed in the receptor number after the TPA treatment (Table 2). These findings suggest that the primary effect of TPA on the adenylate cyclase system may be to uncouple the interaction between the receptor and guanine nucleotide-binding stimulatory regulatory protein of the system, N_s.

Since TPA is known to activate protein kinase C, this kinase may be involved in the above-mentioned finding. 1-Oleoyl-2-acetyl-glycerol (OAG) is also known to activate protein kinase C, and, in addition, it can penetrate cell membrane through lipid bilayer (10,11). As shown in Table 3, treatment of rat reticulocytes with 10 or 100 μM OAG induced the desensitization to isoproterenol but not to NaF-$AlCl_3$. To further ascertain the involvement of protein kinase C in the desensitization induced by TPA, effects of H-7, a potent inhibitor of the kinase (Ki, 6 μM) (12), were examined on the desensitization of adenylate cyclase system of rat reticulocytes to isoproterenol. Fig. 2A

Table 3: Desensitization of adenylate cyclase to a β-adrenergic agonist in OAG-treated reticulocytes

Addition to membrane	Adenylate cyclase activity (pmol/min/mg protein)		
	non-treated	10 μM OAG-treated	100 μM OAG-treated
none	2.2 ± 0.2	2.2 ± 0.2	2.2 ± 0.1
10 μM isoproterenol	40.5 ± 3.6	31.5 ± 2.7	26.5 ± 2.9
5 mM NaF-20 μM $AlCl_3$	26.7 ± 3.0	26.7 ± 2.1	27.0 ± 1.9

Rat reticulocytes were incubated with 10 or 100 μM OAG instead of TPA. Others are the same as in Fig. 1, except for n=3.

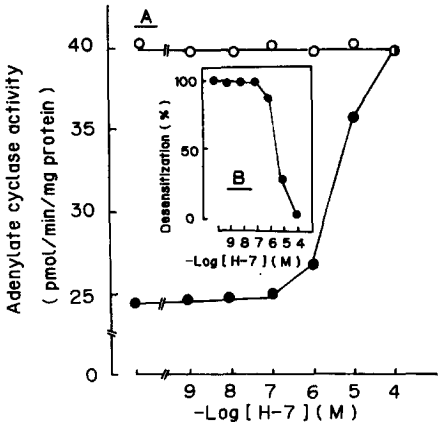

Fig. 2. Preventive effect of H-7 on TPA-induced desensitization to isoproterenol in rat reticulocytes. Panel A: The reticulocytes were incubated with (●) or without (○) 1 μM TPA in the presence of indicated concentrations of H-7 at 30°C for 90 min. Membranes prepared were assayed adenylate cyclase activity in the presence of 10 μM isoproterenol. Panel B: Change in the desensitization versus H-7 concentration, as calculated from values in Panel A.

shows that isoproterenol-stimulated adenylate cyclase activity in the membrane from control reticulocytes was not affected by H-7, while that in the membrane from TPA-treated reticulocytes was markedly increased by H-7 in a dose-dependent manner. At 100 μM H-7, desensitization of the adenylate cyclase activity to isoproterenol by TPA was hardly observed (Fig. 2B). Half maximal concentration of H-7 for the prevention of the desensitization was compatible with above-mentioned Ki value for protein kinase C (12). These results indicate that protein kinase C is involved in a certain type of desensitization to β-adrenergic agents of adenylate cyclase system in rat reticulocytes. Though substrate of protein kinase C in this desensitization process is not clear, phosphorylation of β-adrenergic receptor proteins may be likely, since TPA treatment has no effects on N_s and catalytic unit proteins of this system. Recently, it was reported that isoproterenol-induced desensitization of adenylate cyclase system in avian erythrocytes was closely correlated with the phosphorylation of β-adrenergic receptor, and that cAMP-dependent protein kinase might be involved in the phosphorylation (17). It may be reasonable that various protein kinases are involved in the regulation of adenylate cyclase system through the phosphorylation of different proteins.

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