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ALPHA$_1$ ADRENERGIC RECEPTOR MEDIATED POLYPHOSPHOINOSITIDE BREAKDOWN IN DDT$_1$-MF$_2$ CELLS

LACK OF EVIDENCE OF DESENSITIZATION AFTER PROLONGED EXPOSURE TO EPINEPHRINE

JAN S. ROSENBAUM,* SALMAN AZHAR and BRIAN B. HOFFMAN†

Department of Medicine, Stanford Uiversity School of Medicine, and Geriatric Research, Education and Clinical Center, Veterans Administration Medical Center, Palo Alto, CA 94304, U.S.A.

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Abstract—The DDT$_1$-MF$_2$ cell line is a transformed smooth muscle cell line which is known to possess both alpha$_1$ and beta$_2$ adrenergic receptors. We have utilized these cells to compare the effects of epinephrine pretreatment on the functional capabilities of these two different adrenergic receptors. Pretreatment of the cells grown in suspension with 10^{-7} M epinephrine for 6 hr resulted in desensitization of beta receptor stimulated cyclic AMP accumulation. The maximal response to isoproterenol was decreased to 46 ± 6% of the value in controls ($P < 0.05$); there was also a decrease in the sensitivity of the cells to isoproterenol (log $EC_{50} = -6.65 \pm 0.22$ vs -7.26 ± 0.11 in controls, $P < 0.05$). Also, there was a decrease in the number of beta receptors from 257 ± 29 to 163 ± 22 fmol/mg protein. In contrast, pretreatment with 10^{-6} M epinephrine for 6 hr failed to induce a loss of sensitivity in the ability of the alpha$_1$ receptor agonist phenylephrine to stimulate inositol triphosphate accumulation (log $EC_{50} = -5.59 \pm 0.18$ vs -5.42 ± 0.44 in control cells). A 2-fold increase in basal inositol monophosphate accumulation was observed after epinephrine pretreatment ($P < 0.05$); however, there was no change in maximal phenylephrine-stimulated inositol monophosphate accumulation in these cells. There was a small decrease in the alpha$_1$ receptor number after epinephrine pretreatment ($B_{max} = 457 \pm 89$ fmol/mg protein vs 540 ± 94 in control cells, $P < 0.05$). In contrast to epinephrine pretreatment, pretreatment of cells in suspension with 10^{-7} M 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 15 min resulted in a nearly complete blunting in the ability of both norepinephrine and phenylephrine to stimulate inositol phosphate accumulation: after norepinephrine stimulation, 774 ± 34 dpm in TPA-pretreated cells vs 2590 ± 10 in control cells; inositol monophosphate accumulation after phenylephrine stimulation: 576 ± 25 dpm in TPA-pretreated cells vs 1660 ± 27 in control cells. Basal levels of inositol monophosphate remained unchanged at 544 ± 28 dpm vs 505 ± 31 in TPA-pretreated cells compared to control cells. These data indicate that protein kinase C may exert a negative feedback control on the alpha$_1$ receptor in these cells and that direct activation of protein kinase C by phorbol esters may have a different effect on the alpha$_1$ adrenergic receptor system in DDT$_1$-MF$_2$ cells than does prolonged exposure to epinephrine.

Desensitization involves a decreased responsiveness of a cell or tissue to a challenge by a hormone or drug after previous prolonged exposure of the cell to an agonist [1]. Desensitization has been studied extensively for the beta adrenergic receptor adenylate cyclase system. After prolonged exposure to a beta adrenergic agonist, cells subsequently have a decreased cyclic AMP response when stimulated. This decreased response may be due to a loss in beta adrenergic receptor number due to receptor internalization or a decreased ability of the desensitized receptor to couple to the guanine nucleotide regulatory protein of adenylate cyclase [2, 3].

Much less is known about alpha$_1$ adrenergic receptor desensitization. It is known that functional alpha$_1$ receptor desensitization occurs in vascular smooth muscle after *in vitro* exposure to epinephrine [4–6], as well as after an *in vivo* epinephrine infusion [7], and in rats harboring a pheochromocytoma [8]. In isolated renal tubular epithelial cells, alpha$_1$ receptors have been shown to down-regulate after prolonged exposure to epinephrine [9]; however, the mechanism for the alpha$_1$ receptor mediated desensitization in smooth muscle has not been elucidated. Stimulation of alpha$_1$ receptors activates hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate and diacylglycerol [10] with subsequent mobilization of calcium [11]. However, the role of the phosphatidylinositol system in alpha$_1$ receptor mediated desensitization is not clear.

The DDT$_1$-MF$_2$ cell line is thought to be a transformed smooth muscle cell line which expresses both alpha$_1$ [12, 13] and beta$_2$ adrenergic receptors [14, 15]. The beta$_2$ receptor has been shown to be linked to adenylate cyclase in these cells [15]; the alpha$_1$ receptor has been linked to phosphatidyl-

* Present address: The Proctor & Gamble Co., Miami Valley Laboratories, P.O. Box 39175, Cincinnati, OH 45241.

† Address correspondence and reprint requests to: Brian B. Hoffman, M.D., GRECC (182-B), VA Medical Center, 3801 Miranda Ave., Palo Alto, CA 94304.

inositol hydrolysis in this system [13, 16, 17]. The objective of the current study was to use this cell line as a model system in which to study the possibility of alpha$_1$ receptor mediated desensitization in polyphosphoinositide breakdown. We found, in contrast to other models of desensitization wherein prolonged exposure of a cell to an agonist leads to a blunting in response, an elevation in basal inositol monophosphate accumulation in epinephrine-pretreated cells. This was in marked contrast to the situation observed for the beta receptor adenylate cyclase system in these cells which exhibited the expected blunting in agonist mediated cyclic AMP accumulation after epinephrine pretreatment.

MATERIALS AND METHODS

Materials. [^{3}H]Prazosin and [^{3}H]myo-inositol were purchased from Amersham, Arlington Heights, IL. Phenylephrine HCl was donated by Ciba Geigy (Summit, NJ). [^{125}I[Cyanopindolol was prepared by iodinating cyanopindolol in the presence of chloramine T. [^{125}I] was purchased as the sodium salt from Amersham, and cyanopindolol was a gift of G. Engel. Drugs and chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO) or other commercial sources.

Growth and harvesting. Suspensions of cells were grown to the end of log phase growth (3×10^5 cells/ml) in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 4.5 g glucose/l, penicillin (100 units/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml) and 10% newborn calf serum. Cells were harvested by centrifugation at 100 *g* for 20 min.

Alpha$_1$ and beta adrenergic receptor binding assays. Cells were incubated for 6 hr in the presence of either 10^{-6} M or 10^{-7} M epinephrine (as indicated) and 10^{-4} M ascorbic acid or with 10^{-4} M ascorbic acid alone (controls). To remove epinephrine, cells were washed five times with 25 ml of a buffer (0.14 M NaCl, 0.01 M sodium phosphate, pH 7.2). The final pellet was frozen in liquid nitrogen in 3 ml of buffer (0.25 M sucrose, 1 mM $MgCl_2$, 5 mM Tris, pH 7.4, at 0°) and frozen at −70° prior to use in the binding assays.

Membranes were prepared from frozen cells for use in the binding assay as follows: cells were thawed and lysed in 10 ml buffer (0.01 M Tris, 0.001 M EDTA, pH 7.5) with two 5-sec bursts of a Brinkmann polytron (setting 7) followed by four 5-sec bursts at setting 10. The lysate was centrifuged at 500 *g* for 10 min (4°), and the supernatant fraction was recentrifuged at 39,000 *g* for 30 min, 4°. The final pellet was resuspended in buffer A (0.05 M Tris, 0.01 M $MgCl_2$, pH 7.5, at 25°) using five strokes with a teflon pestle and diluted to a final volume of 0.07 to 0.15 mg protein/ml. The binding assay was performed in a total volume of 2 ml of buffer A containing 1 ml of cell membrane suspension and 25 μl of [^{3}H]prazosin in the presence and absence of 10^{-5} M phentolamine to determine nonspecific binding. Incubations were carried out at 37° for 15 min and were terminated by rapid filtration through Whatman GF/C filters.

Beta adrenergic receptor binding was performed as above except that the binding assay was performed with [^{125}I]cyanopindolol in a total volume of 150 μl which contained 100 μl of a cell suspension diluted to a final protein concentration volume of 0.12 to 0.30 mg/ml.

Stimulation of cyclic AMP accumulation by isoproterenol in DDT$_1$-MF$_2$ cells pretreated with 10^{-7} M epinephrine for 6 hr. Prior to stimulation, cells were harvested and washed as described above for the receptor binding assays. Isoproterenol stimulation of 0.5 to 2×10^6 cells was carried out for 10 min at 37° in DMEM medium containing 0.02 M 4-(2-hydroxyethyl)-1-piperazine-ethanosulfonic acid (HEPES) buffer, penicillin, streptomycin and fungizone in the presence of 5×10^{-4} M isobutylmethylxanthine and 10^{-3} M sodium ascorbate. Cyclic AMP accumulation was determined by radioimmunoassay [18, 19].

Stimulation of inositol phosphate accumulation in DDT$_1$ MF$_2$ cells. To determine the effect of epinephrine pretreatment on the ability of alpha$_1$ receptor agonists to stimulate inositol phosphate accumulation, cells (at a density of 1.5×10^5 cells/ml) were prelabeled with [^{3}H]myo-inositol (5 μCi/ml) 24 hr prior to cell harvesting. Six hours prior to harvesting, cells were treated with 10^{-6} M epinephrine and 10^{-4} M ascorbic acid or 10^{-4} M ascorbic acid alone (control). Cells were harvested as described for receptor binding assays except that the cells were washed in DMEM medium containing penicillin, streptomycin, fungizone, and 0.02 M HEPES buffer. The washing procedure took approximately 1.5 hr and immediately preceded incubation of the cells (0.8 to 2×10^6 cells) with 10 mM LiCl for 15 min prior to addition of phenylephrine. The cells were incubated with phenylephrine for 30 min at 37° in the presence of 10^{-7} M propranolol in a total volume of 1 ml. The reaction was stopped by the addition of 1 ml of 20% trichloroacetic acid (TCA) according to the method of Drummond *et al.* [20]. The inositol phosphates were isolated by ion-exchange chromatography as previously described [20].

Effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) pretreatment on inositol phosphate accumulation. To determine the effect of a direct activator of protein kinase C on the ability of alpha$_1$ receptor agonists to stimulate phosphatidylinositol hydrolysis, DDT$_1$-MF$_2$ cells were prelabeled for 24 hr with [^{3}H]myo-inositol as described above. Cells were harvested, and the free [^{3}H]myo-inositol was removed by washing the cells once in DMEM containing antibiotics, fungizone and 0.02 M HEPES as above. Cells were resuspended at a final density of 2.5×10^6 cells/ml and incubated for 15 min in a 37° shaking water bath with 10^{-7} M TPA. Cells were then incubated with 10 mM LiCl for 15 min prior to the addition of 10^{-3} M phenylephrine or 10^{-5} M norepinephrine in a final value of 1 ml. Stimulation with catecholamine agonists was done in the presence of 10^{-4} M ascorbic acid and 10^{-7} M propranolol. The reaction was stopped by the addition of 1 ml TCA, and analysis of inositol phosphates proceeded as above.

Data analysis. Receptor binding data were analyzed using the LIGAND program [21] on an HP9816 computer. All dose–response curves were analyzed using a version of the ALLFIT program [22] modified

Table 1. Alpha$_1$ and beta receptor binding in control and epinephrine-pretreated DDT$_1$-MF$_2$ cells

	Alpha$_1$ receptors		Beta receptors	
		[^{3}H]Prazosin		[^{125}I]Cyanopindolol
Group	B_{max} (fmol/mg protein)	K_D (M)	B_{max} (fmol/mg protein)	K_D (M)
Control	540 ± 94	6.0 ± 0.7 × 10^{-11}	257 ± 29	3.4 ± 0.4 × 10^{-11}
Epinephrine-pretreated	457 ± 89*	7.5 ± 0.4 × 10^{-11}	163 ± 22*	2.8 ± 0.5 × 10^{-11}

Cells were preincubated for 6 hr with 10^{-6} M epinephrine in the alpha$_1$ receptor experiments and with 10^{-7} M epinephrine in the beta receptor experiments. Values are means ± SEM; N = 6 for the alpha$_1$ receptor experiments and N = 4 for the beta receptor experiments.

* Significantly different from control, $P < 0.05$.

for the Apple II system by Martin H. Teicher. These data analysis programs were obtained from the Biomedical Computing Technology Information Center (Vanderbilt Medical Center, Nashville, TN). Statistical analysis was performed using the paired two-tailed Student's *t*-test.

RESULTS

Alpha$_1$ adrenergic and beta adrenergic receptor number after pretreatment of DDT$_1$-MF$_2$ cells with epinephrine for 6 hr. Cells were preincubated with 10^{-6} M epinephrine in the alpha$_1$ receptor experiments and 10^{-7} M in the beta adrenergic receptor studies. The higher concentration of epinephrine was used in the alpha receptor studies since preliminary experiments showed an absence of change in receptor number with 10^{-7} M epinephrine (data not shown). The results from the receptor binding experiments in control and epinephrine-pretreated cells are shown in Table 1. A small but statistically significant down-regulation of alpha$_1$ receptors was found in the epinephrine-pretreated cells, whereas the down-regulation of beta receptors was more marked. There were no changes in the K_D values of [^{3}H]prazosin or [^{125}I]cyanopindolol.

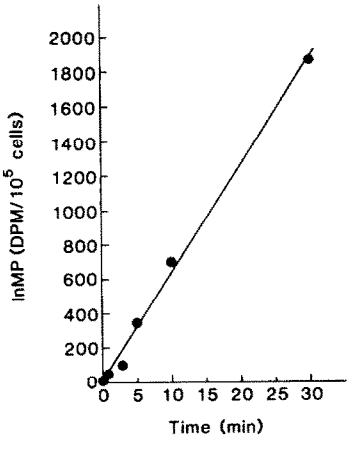

Fig. 1. Time course of phenylephrine-stimulated inositol-1-monophosphate accumulation. Cells were stimulated with 10^{-5} M phenylephrine in the presence of 10 mM LiCl for the indicated times, and inositol-1-monophosphate (InMP) was measured as indicated in Materials and Methods. Results represent the mean of duplicate determinations. The experiment was repeated once with similar results.

Stimulation of inositol phosphate accumulation in DDT$_1$-MF$_2$ cells by alpha adrenergic agonists. Figure 1 illustrates the time course for the accumulation of inositol-1-monophosphate by 10^{-5} M phenylephrine in the presence of 10 mM LiCl. As can be seen, the time course was linear over 30 min ($r = 0.99$), indicating that a rapid desensitization of alpha$_1$ receptors did not occur in these cells. Consequently, an incubation time of 30 min was chosen for subsequent studies of alpha$_1$ receptor stimulation. Figure 2 illustrates the phenylephrine dose–response curve for the stimulation of inositol-1-monophosphate (InMP) accumulation in control and epinephrine-pretreated cells. Analysis of four similar experiments revealed no change in the EC_{50} value of phenylephrine in the epinephrine-pretreated cells (Table 2). While the maximal response appeared elevated in epinephrine-pretreated cells, this difference was not statisically significant. However, the basal activity was increased significantly in the cells pretreated with epinephrine (Table 2). The apparent decrease in fold stimulation in epinephrine-pretreated cells was therefore due to the increased basal response. Similar results were obtained for inositol 1,4-diphosphate (InDP) and inositol 1,4,5-triphosphate (InTP); also, similar results were obtained when the cells were prelabeled with [^{3}H]myo-inositol for 48 hr rather than 24 hr (data not shown).

Stimulation of inositol phosphate accumulation by alpha adrenergic agonists in cells pretreated with TPA. Figure 3 illustrates the effect of pretreating DDT$_1$-MF$_2$ cells with 0.1 μM TPA on the ability of phenylephrine and norepinephrine to stimulate inositol monophosphate, inositol 1,4-diphosphate and inositol 1,4,5-triphosphate accumulation. Inositol monophosphate accumulation was stimulated more by 10^{-5} M norepinephrine than by 10^{-3} M phenylephrine. This result suggests that phenylephrine may be a partial agonist at alpha$_1$ receptors in DDT$_1$-MF$_2$ cells. Pretreatment of the cells with TPA for only 15 min resulted in a nearly complete blunting of both phenylephrine- and norepinephrine-mediated inositol monophosphate accumulation (Fig. 3). This result was found for each of the individual inositol phosphate pools. TPA pretreatment did not affect basal levels ($P \geq 0.05$). Similar results

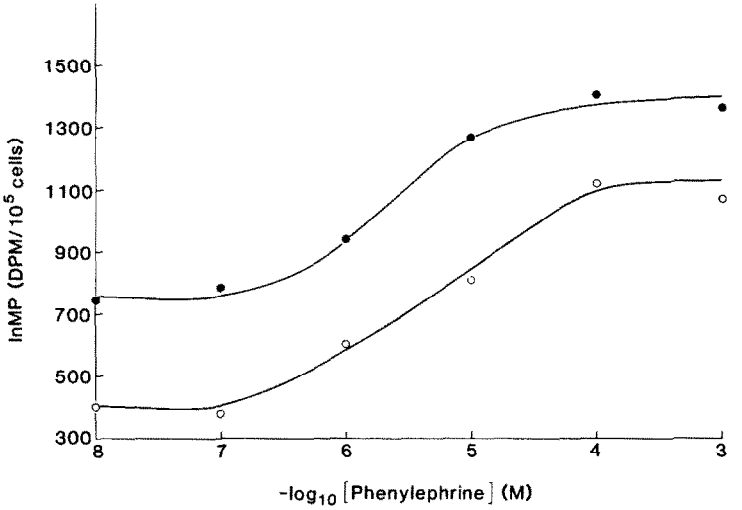

Fig. 2. Phenylephrine dose–response curve for the stimulation of inositol-1-monophosphate accumulation in control and epinephrine-pretreated cells. After epinephrine pretreatment (10^{-6} M for 6 hr) and extensive washing, cells were stimulated with phenylephrine for 30 min after a 15-min preincubation with 10 mM LiCl. The accumulation of inositol-1-monophosphate (InMP) was determined as described in Materials and Methods. Results represent the mean of duplicate determinations. Basal levels of inositol-1-monophosphate were 397 dpm/10^5 cells in control (○) and 775 cpm/10^5 cells in epinephrine-pretreated (●) cells. Basal levels were unaffected by the addition of 10^{-6} M prazosin, indicating that the increased basal levels in epinephrine-pretreated cells were not due to the presence of retained epinephrine. The experiment was repeated three times with similar results.

were observed when the cells were prelabeled with $^{32}P_i$, and the individual phosphatidylinositide pools were separated and analyzed (data not shown).

Desensitization in beta adrenergic receptor mediated cyclic AMP accumulation production in epinephrine (10^{-7} M) pretreated DDT_1-MF_2 cells. Figure 4 illustrates the effect of pretreatment of the cells with 10^{-7} M epinephrine for 6 hr on the beta adrenergic receptor mediated stimulation of cyclic AMP accumulation. Desensitization of beta adrenergic receptors was evidenced by both an increase in the EC_{50} value for isoproterenol and a decreased maximal response ($P < 0.05$) (Table 3). There was no change in basal levels of cAMP production in epinephrine-pretreated cells.

DISCUSSION

We have demonstrated that, while there was typical beta receptor densensitization in DDT_1-MF_2 cells exposed to epinephrine, the alteration in $alpha_1$ responsiveness was quite different in these cells. Exposure of these cells to 10^{-7} M epinephrine for 6 hr was sufficient to induce a blunting in the beta receptor stimulated increase in cAMP as well as down-regulation of the beta receptors (this study and Ref. 14). In contrast, exposure of the cells to a 10-fold higher epinephrine concentration for the same period was insufficient to induce either a blunting in the $alpha_1$ receptor mediated inositol monophosphate accumulation or a loss in agonist sensitivity. Rather, an increase in the basal inositol monophosphate accumulation was observed. Also, epinephrine pretreatment led to a modest down-regulation of the $alpha_1$ receptor number.

The finding of beta adrenergic receptor desensitization (Fig. 4) in these cells is in agreement with the findings of Scarpace *et al.* [14] and indicates that the cells are capable of undergoing a desensitization characterized by a decrease in maximal response and

Table 2. Characterization of the phenylephrine dose–response for inositol-1-monophosphate accumulation in control and epinephrine-pretreated cells

Group	Log EC_{50}	Normalized Basal	Normalized E_{max}	Fold stimulation (E_{max}/Basal)
Control	−5.420 ± 0.436	1.00 ± 0	1.00 ± 0	2.96 ± 0.21
Epinephrine-pretreated	−5.592 ± 0.181	2.14 ± 0.29*	1.24 ± 0.19	1.71 ± 0.11†

Cells were pretreated for 6 hr with 10^{-6} M epinephrine prior to harvesting and stimulation. Data are normalized to the basal or maximal dpm values determined by the ALLFIT program [22] for the control cells. The resulting normalized values were then averaged and presented as means ± SEM of four experiments. Representative values are illustrated in Fig. 2. Similarly, the fold stimulation values were determined using the fitted values for E_{max} (maximal response) and basal levels determined by ALLFIT.

* Significantly different from control, $P < 0.05$.

† Significantly different from control, $P < 0.01$.

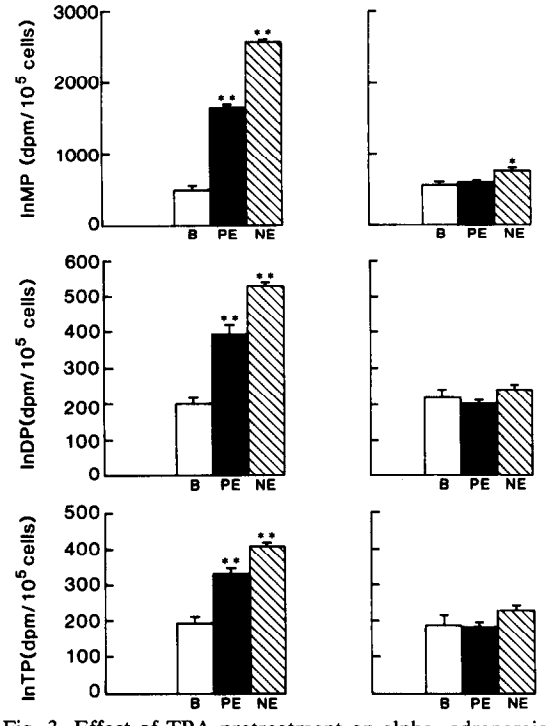

Fig. 3. Effect of TPA pretreatment on alpha$_1$ adrenergic receptor mediated inositol phosphate accumulation. Control cells (left panels) or those pretreated with 10^{-7} M TPA (right panels) for 15 min were stimulated with either 10^{-3} M phenylephrine or 10^{-5} M norepinephrine after a 15-min preincubation with 10 mM LiCl as indicated in Materials and Methods. Basal levels were not different in the TPA-pretreated cells. The results represent the mean ± SEM of two experiments, each performed in duplicate. Key: statistically different from basal; (*) $P < 0.05$, and (**) $P < 0.01$.

a rightward shift in the agonist dose–response curve. Such a pattern of desensitization may be due to both a receptor down-regulation, which has been shown previously been shown to occur in these cells for the beta receptor [14], and a decreased ability of the receptor to couple to the guanine nucleotide binding protein of adenylate cyclase [2, 3].

The effects of epinephrine pretreatment on alpha$_1$ adrenergic receptor number and responsiveness stand in contrast to the situation observed for the beta adrenergic receptor. There was a more marked down-regulation of beta receptors in these cells compared to the down-regulation of alpha$_1$ receptors. A decrease in alpha$_1$ receptor number after prolonged exposure to catecholamine agonists has been observed in other cell systems [9, 23]. However, it is possible that we may have underestimated the extent of down-regulation since it took approximately 1.5 hr of washing to remove completely the free epinephrine from the cells; during that time it is possible that some of the down-regulation could have reversed.

It is known that epinephrine pretreatment of rabbit aorta ring segments *in vitro* results in a blunting of the alpha adrenergic receptor mediated stimulation of phosphatidylinositol turnover [6] and vascular smooth muscle contraction. However, in the DTT$_1$-MF$_2$ cells exposed to epinephrine we found an absence of change in the maximal alpha$_1$ receptor mediated stimulation of inositol monophosphate accumulation. Also, there was a marked increase in basal inositol monophosphate accumulation in epinephrine-pretreated DDT$_1$-MF$_2$ cells (Fig. 2). The explanation for the increase in basal levels of inositol monophosphate is unclear; to our knowledge, this response to prolonged alpha$_1$ stimulation has not been seen previously. To what extent the

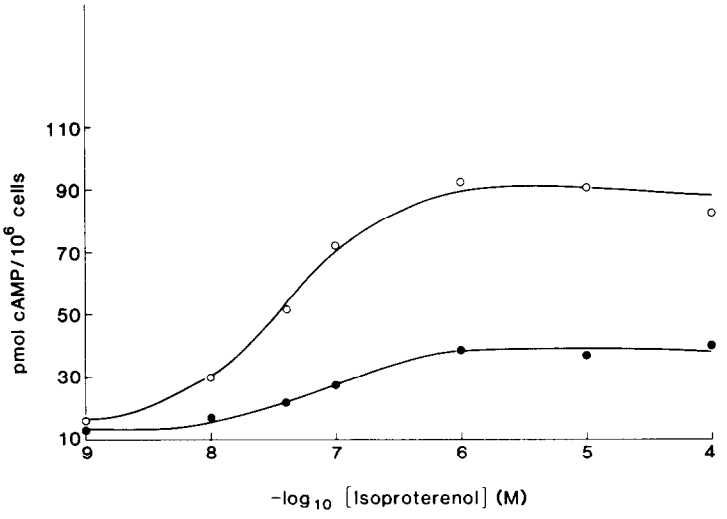

Fig. 4. Isoproterenol dose–response curve for the stimulation of cAMP accumulation in control and epinephrine-pretreated cells. After epinephrine pretreatment (10^{-7} M for 6 hr) cells were washed extensively and then stimulated with isoproterenol for 10 min. Basal levels of cAMP were 11.48 ± 1.24 pmol/10^6 cells in control (N = 4) (○) and 13.72 ± 1.76 pmol/10^6 cells in epinephrine-pretreated (N = 4) (●) cells. Basal levels were not significantly different and were unaffected by the addition of 10^{-5} M propranolol, indicating that the epinephrine was effectively washed out of the pretreated cells.

Table 3. Characterization of the isoproterenol dose–response for cyclic AMP accumulation in control and epinephrine-pretreated cells

Group	Log EC_{50}	Normalized basal	Normalized E_{max}	Fold stimulation (E_{max}/Basal)
Control	−7.258 ± 0.110	1.00 ± 0	1.00 ± 0	6.53 ± 0.59
Epinephrine-pretreated	−6.646 ± 0.215*	0.98 ± 0.18	0.46 ± 0.06*	3.11 ± 0.07*

Cells were pretreated for 6 hr with 10^{-7} M epinephrine prior to harvesting and stimulation. Normalized values were obtained as in the legend to Table 2. Values are means ± SEM of three experiments. Representative values for E_{max} and basal levels are indicated in Fig. 4.

* Significantly different from control, $P < 0.05$.

washing of the cells after the epinephrine pretreatment may have allowed recovery of the $alpha_1$ receptor mediated response could not be determined in the design of these experiments.

Our finding of an effect of TPA pretreatment on the $alpha_1$ adrenergic receptor mediated polyphosphoinositide hydrolysis supports observations made by others in this cell line [16, 17] and in other preparations [24, 25]. These data indicate that TPA, possibly by activating C kinase, induces a blunting in $alpha_1$ adrenoceptor mediated polyphosphoinositide hydrolysis. It is interesting that a blunting in response is observed after phorbol ester pretreatment, whereas it is not observed after pretreatment of the cells with epinephrine. It has been proposed that protein kinase C, the enzyme which is activated by TPA [26], may exert negative feedback control over the $alpha_1$ adrenergic receptor [27]. This negative feedback control of the $alpha_1$ receptor may be responsible for the blunting in the ability of both phenylephrine and norepinephrine to stimulate polyphosphoinositide metabolism after TPA treatment of the cells. Furthermore, this deactivation of the $alpha_1$ receptor by TPA is associated with phosphorylation of the $alpha_1$ receptor [16]. If receptor phosphorylation is necessary for inactivation of the $alpha_1$ receptor in these cells, our data suggest that activation of protein kinase C by phorbol esters is a more effective mechanism for inactivating the $alpha_1$ receptors than is continued exposure of the cells to a high concentration of an adrenergic receptor agonist.

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