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ACCELERATED COMMUNICATION

Identification of a Specific Domain in the β-Adrenergic Receptor Required for Phorbol Ester-Induced Inhibition of Catecholamine-Stimulated Adenylyl Cyclase

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SUMMARY

The molecular basis for the effects of 4β-phorbol 12-myristate 13-acetate (PMA) on adenylyl cyclase activation was examined using site-directed mutants of the hamster β-adrenergic receptor (βAR) expressed in L cells. Phorbol ester activation of protein kinase C (PKC) in L cells transfected with wild-type βAR caused at least three discernible effects on adenylyl cyclase activity, each with an EC_{50} of 20 to 50 nM, (i) a 2–3-fold increase in the K_{act} for epinephrine stimulation, (ii) a 2–3-fold increase in the maximal level (V_{max}) of hormonal stimulation, and (iii) a decrease in the G_i-mediated inhibition of forskolin stimulation. Deletion from the βAR of amino acid residues 259–262, which removes one of the two consensus sites for phosphorylation by PKC, eliminated (>90%) the PMA-induced increase in the K_{act}, whereas the PMA-induced increase in the V_{max} and loss of G_i-mediated inhibition were not affected by the deletion. Neither deletion of the other PKC consensus site in the βAR (residues 343–348) nor truncation of the Ser/Thr-rich C-terminal domain (residues 354–418) affected the PMA-induced changes in adenylyl cyclase. The effects of PMA on G_i-mediated inhibition and the V_{max} closely mimicked the action of islet-activating protein, consistent with a direct effect of PMA-activated PKC on G_i. In contrast, the effects on the K_{act} appear to be receptor specific. These results demonstrate that the consensus site for phosphorylation by PKC, found in the third intracellular loop of the βAR, is required for the PMA-induced increase in the K_{act} for epinephrine stimulation. Use of L cells transfected with D(259–262)βAR allowed the characterization of the postreceptor effects of PMA without interference from receptor-level effects.

Given the complexity of the regulation of cellular processes by the second messengers cAMP, Ca^{2+}, and diacylglycerides, it is not surprising that extensive cross-regulation of their levels in cells has been observed. Some insight into the extent of this cross-regulation has resulted from studies of the action of phorbol esters on the hormonal regulation of adenylyl cyclase (1–7). It is now generally accepted that the primary, if not sole, mechanism of action of the phorbol esters is through activation of various isozymes of PKC. Because it appears that activation of PKC by PMA is very similar to activation by diacylglycerides, it is presumed that the effects of PMA on adenylyl cyclase mimic in part the action of hormones that activate phospholipase C.

In a number of cell types, it has been shown that activation of PKC by phorbol esters causes both receptor-specific inhibition of hormonal stimulation of adenylyl cyclase activation, characterized by a decrease in the potency of agonist stimulation, and a more general augmentation of hormonal stimulation, which is associated with the loss of G_i-mediated inhibition (1, 2, 4–6). Because the receptor- and G_i-specific actions of PMA have opposite effects on adenylyl cyclase activity, it has been difficult to discern the molecular basis of these effects. Studies of the avian erythrocyte suggest that the decrease in the potency of βAR activation of adenylyl cyclase following PMA-induced desensitization is caused by phosphorylation of the receptor (1, 2, 8); however, the domains of the βAR that are phosphorylated in response to PMA have not been identified.

The augmentation of hormonal stimulation and loss of G_i-mediated inhibition following PMA treatment of cells have been variously attributed to phosphorylation and inactivation of G_i by PKC (4–6) or to phosphorylation of adenylyl cyclase

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ABBREVIATIONS: PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate; βAR, β-adrenergic receptor; cAPK, cAMP-dependent protein kinase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IAP, islet-activating protein.

itself (7). IAP, which acts by ADP-ribosylation of G_i, has effects on G_i function similar to those of PMA in the S49 wild-type and cyc$^-$ lymphoma cell lines, consistent with a PMA-mediated modification of G_i (6); however, although there have been reports that PKC may cause phosphorylation of G_i in hepatocytes, evidence is confusing and preliminary (9). In platelets, the α subunits of $G_{i\ 1-3}$ do not appear to be directly phosphorylated following treatment with PMA, despite impaired G_i function; however, phosphorylation of $G_r\alpha$, a M_r 40,000 GTP-binding protein whose function remains unknown, has been observed by Carlson *et al.* (10). In addition, Yoshimasa *et al.* (7) have reported that phorbol ester-induced phosphorylation of a M_r 130,000 protein that binds to a forskolin-Sepharose affinity column and was tentatively identified as adenylyl cyclase.

Following the cloning of the hamster βAR (11–14), it was recognized that there are two consensus sites for both cAPK and PKC in the βAR, one on the third intracellular loop (Ser^{262} for cAPK and Ser^{261} for PKC) and another in the C-terminal domain (Ser^{346} for cAPK and PKC). These consensus sites were identified on the basis of criteria established by Krebs and coworkers (15–17) for cAPK and by Woodgett *et al.* (18) for PKC. We have previously demonstrated that the consensus site involved in cAPK-mediated desensitization of the βAR is Ser^{262} on the third intracellular loop of the βAR, because a deletion mutant of the hamster βAR lacking amino acids 259–262 was unable to undergo cAPK-mediated desensitization (19). Deletion of the C-terminal site (residues 343–348) or truncation of the C-terminal tail did not alter the cAPK-mediated desensitization (19).

In the present study, we have used mutagenesis of the hamster βAR to determine whether either of the PKC consensus sites on the receptor is required for any of the pleiotropic effects of phorbol esters on activation of adenylyl cyclase by adrenergic agonists. Amino acids 259–262 within the third intracellular loop were found to be critical for the PMA-induced decrease in the potency of βAR activation of adenylyl cyclase, whereas residues within the C-terminal domain were not involved. The use of the mutant receptor lacking the PKC consensus site in the third intracellular loop has enabled us to separate the receptor-specific from the downstream effects of phorbol esters. These studies suggest that inactivation of G_i is a likely mechanism for the postreceptor effects of PMA.

Materials and Methods

Cell culture. Mouse L cells were transfected with hamster βARs, as previously described (14). Cells expressing the wild-type βAR are designated as such. The cell lines D(259–262)βAR and D(343–348)βAR are deletion mutants expressing βARs lacking the amino acid residues in parentheses. The truncation mutant T(354)βAR is missing the entire C-terminal tail from amino acids 354 to 418. The cells were cultured at 37° in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM L-glutamine, and 0.4 mg/ml Geneticin.

Cell incubations and membrane preparations. Cells were treated at 37° for 10 min with dimethyl sulfoxide or PMA, such that the final concentration of dimethyl sulfoxide was 0.2%. The cells were then washed twice with cold HME buffer (20 mM HEPES, pH 8, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM benzamidine, 2 mM tetrasodium pyrophosphate, 10 μg/ml trypsin inhibitor, and 0.1 mg/ml bovine serum albumin), scraped in HME plus 10 μg/ml leupeptin, and homogenized with five strokes of a type B dounce homogenizer. Membranes were then prepared over sucrose gradients as previously described (19).

For the pertussis toxin experiments, IAP from List Biological Laboratories was suspended in 0.1 M sodium phosphate, pH 7, 0.5 M NaCl, to a concentration of 10 μg/ml. Cells were treated for 16–18 hr before the PMA treatment with 10–20 ng/ml IAP or an equal volume of the buffer.

Adenylyl cyclase assays. Adenylyl cyclase was assayed as previously described (19). The free Mg^{2+} concentration, unless otherwise indicated, was 0.3 mM, as calculated by the method of Iyengar and Birnbaumer (20). Other assay components were 40 mM HEPES, pH 7.7, 1 mM EDTA, 8 mM creatine phosphate, 16 units/ml creatine kinase, 0.2 mM ATP, 1 μM GTP, 0.1 mM 1-methyl-3-isobutylxanthine, and 2 μCi of [α-^{32}P]ATP. In experiments where GTP-mediated inhibition of forskolin stimulation was assayed, GTP was added to the specified concentrations.

Results

Characterization of the effects of PMA pretreatment on the βAR stimulation of adenylyl cyclase. To determine which domains of the βAR are involved in the effects of PMA, various L cell clones stably transfected with either the wild-type or mutant βARs were treated for 10 min with concentrations of PMA ranging from 20 nM to 2 μM. Membranes of these cells were used for measurements of epinephrine stimulation of adenylyl cyclase. The results of a typical experiment in cells expressing the wild-type βAR are shown in Fig. 1. PMA treat-

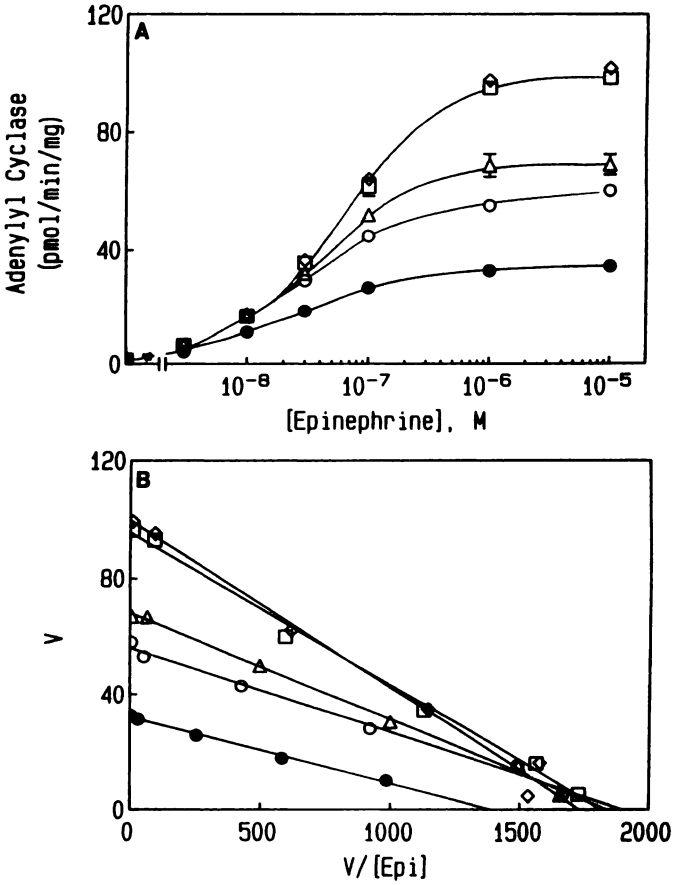

Fig. 1. Dose response of PMA treatment of L cells expressing wild-type βAR. Cells were treated for 10 min with 0.2% dimethyl sulfoxide (●) or 20 nM (○), 50 nM (△), 500 nM (□), or 2 μM (◇) PMA, and membranes were prepared. Adenylyl cyclase was measured at various epinephrine concentrations, and those activities are shown in A. The data shown are mean ± standard deviation of triplicate determinations from a typical experiment. The Eadie-Hofstee transformation of the data appears in B.

ment at a concentration of 20 nM almost doubled the V_{max} for epinephrine stimulation of adenylyl cyclase. Treatment with increasing concentrations of PMA caused progressive increases in both the V_{max} and K_{act} for epinephrine stimulation (see Eadie-Hofstee plot in Fig. 1B). Concentrations of PMA equal to or greater than 0.5 μM produced, on average, a 2.8-fold increase in the V_{max} and a 2.7-fold increase in the K_{act} (Table 1). Treatment of cells expressing D(343–348)βAR or T(354)βAR with 0.5 μM PMA caused increases in both the K_{act} and V_{max} for epinephrine stimulation, which were similar to those of the wild-type βAR (Table 1). Of interest, similar increases in the V_{max} and K_{act} for prostaglandin E_1-stimulated adenylyl cyclase were observed in L cell membranes isolated from PMA-treated cells (data not shown).

In contrast to these results, incubation of the L cells expressing D(259–262)βAR with 0.5 μM PMA did not cause a significant increase in the K_{act} for epinephrine stimulation of adenylyl cyclase (Table 1). However, the increase in the V_{max} for epinephrine stimulation of adenylyl cyclase was similar to that observed for the wild-type βAR (3.6-fold). Similar results were obtained following treatment of the double mutant D(259–262)D(343–348)βAR (data not shown).

Effect of Mg^{2+} on the PMA-induced increase in K_{act} and V_{max}. In the experiments shown in Fig. 1 and Table 1, adenylyl cyclase was measured with a free Mg^{2+} concentration of 0.3 mM. We have previously shown that this concentration of Mg^{2+} is necessary for observation of significant decreases in the potency of epinephrine stimulation of adenylyl cyclase caused by incubation of S49 wild-type lymphoma cells with either PMA or epinephrine (6, 21, 22). As with the S49 lymphoma cells, 10 mM Mg^{2+} concentrations considerably diminished the PMA-induced difference in the K_{act} for epinephrine stimulation. In L cells expressing the wild-type βAR, the average fold increase in K_{act} caused by PMA was reduced from 2.7 (Table 1) with 0.3 mM Mg^{2+} to 1.6 ± 0.3 (three experiments) with 10 mM Mg^{2+}. The PMA-induced increase in the V_{max} for epinephrine-stimulated adenylyl cyclase was eliminated by 10 mM Mg^{2+}; i.e., the fold increase was reduced from 2.8 (Table 1) with 0.3 mM free Mg^{2+} to 1.0 ± 0.06 (three experiments) with 10 mM Mg^{2+}. Elevated concentrations of Mg^{2+} also eliminated the PMA-induced increase in the V_{max} in membranes from L cells expressing the D(259–262)βAR.

Effects of IAP pretreatment on the PMA-induced changes in kinetic parameters. Previous studies of the PMA-induced augmentation of epinephrine stimulation of adenylyl cyclase from S49 lymphoma cells were confounded by the fact that the increase in the V_{max} of epinephrine stimulation was small and that it could not be separated from the effects of the increase in the K_{act}. The substantial increase in the V_{max} we routinely observe in the L cell clones and the potential for isolating G_i-specific effects in cells expressing D(259–262)βAR prompted us to examine the effect of IAP on the PMA-induced augmentation of epinephrine stimulation. If IAP and PMA both blocked G_i function, we reasoned that their effects on the augmentation of epinephrine stimulation and GTP inhibition should be similar and not additive at maximally effective concentrations. As shown in Fig. 2A, IAP treatment increased the V_{max} for epinephrine stimulation of adenylyl cyclase in membranes from cells expressing the wild-type βAR but did not alter the K_{act} for epinephrine stimulation. The combined effects of PMA and IAP on the V_{max} were not additive, although some additional augmentation was usually observed with the two agents in combination. The increased K_{act} caused by PMA treatment of the wild-type βAR was not altered by pretreatment with IAP (Fig. 2A). In contrast, the effects of PMA and IAP on cells expressing D(259–262)βAR were nearly identical, because PMA did not alter the K_{act} in these cells (Fig. 2B). Both IAP and PMA increased the V_{max} for epinephrine stimulation of adenylyl cyclase. The combination of PMA and IAP produced a slightly greater effect than either alone, but it was clearly not additive.

GTP inhibition of forskolin stimulation: comparison of PMA and IAP treatment of L cells expressing wild-type βAR and the D(259–262)βAR. One of the characteristics of PMA treatment of cells is the loss of G_i-mediated inhibition of adenylyl cyclase activity (4–6), and it has been suggested that the observed augmentation of epinephrine stimulation is caused by this loss. Because the effect of IAP on the augmentation of epinephrine stimulation was very similar to that of PMA, we thought that their effects on GTP inhibition of forskolin stimulation would be nearly identical. However, there were some differences, as can be seen in Fig. 3. IAP caused the complete loss of GTP inhibition of forskolin activation of adenylyl cyclase from cells expressing the wild-type βAR, whereas PMA, while causing a substantial loss of the GTP inhibition, was less effective than IAP. Similar results were obtained with the D(259–262)βAR (data not shown). In addition, IAP treatment caused a 2–3-fold decrease in forskolin activation in the absence of any added GTP, whereas PMA had no significant effect on this activity.

TABLE 1

Effect of 0.5 μM PMA treatment on epinephrine stimulation of adenylyl cyclase

Intact transfected L cells were treated for 10 min at 37° with 0.2% dimethyl sulfoxide (DMSO) or 0.5 μM PMA in dimethyl sulfoxide. Membranes were prepared as described in Materials and Methods and were assayed for epinephrine-stimulated adenylyl cyclase. The values are mean ± standard error of the number of experiments shown. The numbers in parentheses are the fold increase of PMA, treated over control values; *n* = number of experiments.

Cell line	V_{max}		K_{act}	
	DMSO	PMA	DMSO	PMA
	pmol/min/mg		nM	
Wild-type βAR (*n* = 6)	39.3 ± 9.9	109.1 ± 19.3 (2.8)	26.1 ± 6.4	71.2 ± 17.6 (2.7)
D(259–262)βAR (*n* = 5)	42.1 ± 4.3	149.8 ± 24.3 (3.6)	26.7 ± 3.0	32.5 ± 4.4 (1.2)
T(354)βAR (*n* = 3)	6.0 ± 1.6	31.3 ± 1.7 (5.2)	9.1 ± 2.7	29.3 ± 7.0 (3.2)
D(343–348)βAR (*n* = 3)	46.8 ± 7.5	132.6 ± 20.9 (2.8)	13.4 ± 1.7	30.3 ± 3.2 (2.3)

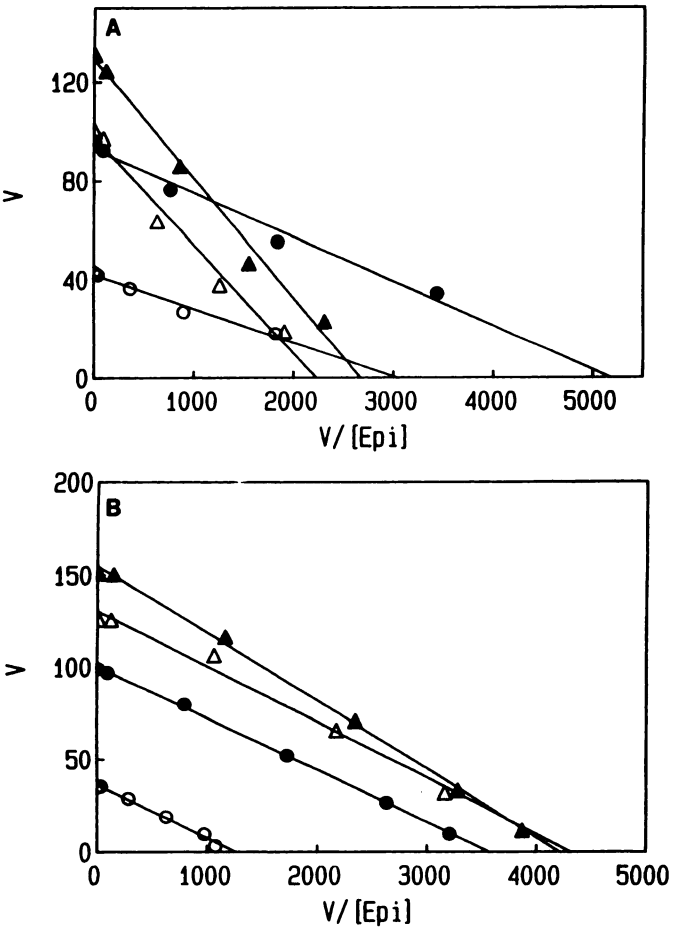

Fig. 2. Effects of IAP treatment on PMA-induced changes in V_{max} and K_{act}. L cells expressing wild-type βAR (A) or D(259–262)βAR (B) were pretreated with (●, ▲) or without (○, △) 20 ng/ml IAP overnight, as described in Materials and Methods. Cells were subsequently treated for 10 min with dimethyl sulfoxide (○, ●) or 0.5 μM PMA (△, ▲), and membranes were prepared and assayed for epinephrine stimulation of adenylyl cyclase. Data are presented as the Eadie-Hofstee transformation.

Discussion

The criteria for establishing the mechanism of regulation of a cellular process by a protein kinase have been expressed clearly by Krebs and Beavo (23). One of the important criteria is that the site or sites of phosphorylation of the protein in question be demonstrated in the intact cell. The low levels of βAR in mammalian cells have so far precluded the determination of the sites that are phosphorylated by protein kinases, using classical protein chemistry. In the present work, we have used the techniques of site-directed mutagenesis to establish the domain of the βAR involved in the action of PMA/PKC on epinephrine stimulation of adenylyl cyclase. Deletion of the PKC consensus site in the third intracellular loop of the βAR (Arg^{259}-Arg-Ser-Ser^{262}) prevented the receptor-specific decrease in the potency of epinephrine stimulation that is associated with receptor desensitization. This modification of the βAR did not alter the other effects of PMA/PKC on adenylyl cyclase, namely the augmentation of activity and the loss of G_i-mediated inhibition. This mutation of the βAR does not affect ligand binding or the coupling efficiency of the receptor to G_s, although the N-terminal and C-terminal domains of this third intracellular loop are required for G_s activation (24). Thus, it appears

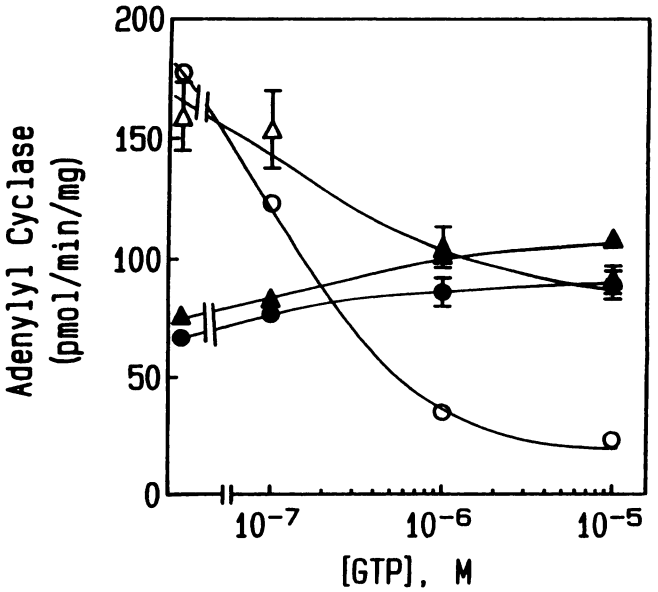

Fig. 3. Effects of IAP and PMA treatment of cells on GTP inhibition of forskolin stimulation. L cells expressing wild-type βAR were treated with (●, ▲) or without (○, △) IAP, and then with (△, ▲) or without (○, ●) PMA, as described in the legend to Fig. 2. The ability of 20 μM forskolin to stimulate adenylyl cyclase in the presence of various concentrations of GTP was assessed. The data shown are the mean ± standard error of two experiments, each assayed in triplicate.

likely that the effect of the deletion was specific and not attributable to a secondary effect such as a generalized conformational change in the receptor. Our results, however, can not completely rule out the possibility that deletion of amino acid residues 259–262 results in a conformational change in the βAR, preventing the interaction of PKC with the receptor.

Our data also demonstrated that the other consensus site for PKC in the C-terminal domain of the hamster βAR (Arg^{343}-Arg-Ser-Ser-Ser-Lys^{348}) was not involved in the action of PMA, because deletion of this site from the receptor had no effect on any of the parameters of PKC-mediated action. In studies on isolated peptides corresponding to these two PKC/cAPK consensus sites, Blake *et al.* (25) observed that the peptide corresponding to the C-terminal PKC site was in fact a better site for PKC phosphorylation *in vitro* than was the peptide corresponding to the site in the third loop of the βAR. However, the results of the current study suggest that, in the native receptor, the relevant site is in the third loop of the βAR and the C-terminal site is not involved. The data do not, however, rule out a more localized indirect (conformational) effect as a result of the deletion. Our results also indicated that the extensive array of serines and threonines in the C-terminal tail of the βAR are not involved in the PMA-induced decrease in agonist potency, because the truncation mutant T(354)βAR also behaved like the wild-type βAR. Although these data seem conclusive, it should be noted that we have not yet examined the effects of prolonged activation of PKC by PMA. It is possible that this could lead to modification of the C-terminal consensus site, which could have additional effects on receptor/adenylyl cyclase coupling that are not evident in our short term treatments.

Our earlier studies of PMA-induced desensitization of the S49 and DDT_1MF-2 cells (22) showed that high free Mg^{2+} levels considerably reduced the increase in the K_{act} for epinephrine stimulation, whereas physiological levels (26, 27) of free

Mg^{2+} (0.1–0.4 mM) allowed its measurement. Our present data confirm this finding. In addition, we discovered that the PMA-induced sensitization of epinephrine stimulation of adenylyl cyclase in L cells (increased V_{max}) was essentially eliminated by 10 mM free Mg^{2+}. In earlier studies of the S49 lymphoma cells, we had been unable to determine whether Mg^{2+} affected the PMA-induced increase in the V_{max}. However, the larger increase in the V_{max} in response to PMA treatment of the L cells, as well as the availability of the D(259–262)βAR, allowed unambiguous interpretation of the effect of Mg^{2+} on the V_{max}, because there was no receptor-level inhibitory effect. Our results suggest that it is crucial to measure adenylyl cyclase activity with low free mg^{2+} and that the high concentrations used by many investigators likely obscure or greatly reduce the increases in both the K_{act} and the V_{max} caused by PMA.

Although we now have good evidence concerning the mechanism of the PMA-induced action at the receptor level, the mechanism involved in the PMA-mediated augmentation of hormonal activation of adenylyl cyclase remains unclear. However, the D(259–262)βAR mutant now gives us a means to study the augmentation of hormonal stimulation without the interference of the receptor-level alterations that in previous studies of the S49 adenylyl cyclase (6) were vexing. In addition, the results in Fig. 2 demonstrated, with a clarity not possible in the S49 cells, that the PMA-induced augmentation of epinephrine-stimulated adenylyl cyclase activity is in fact very similar (although not identical) to that caused by IAP. The PMA-induced augmentation is unrelated to the action of PMA on the βAR and is consistent with a PKC-mediated blockade of G_i function caused by the phosphorylation of G_i, phosphorylation of the catalyst of adenylyl cyclase (7), or both.

Elucidation of the molecular actions of PMA on G_i function will require (i) identification of the G_i that mediates the inhibition of adenylyl cyclase, (ii) determination of whether G_i is phosphorylated or otherwise modified (it is most likely a covalent alteration because the effect is stable), and (iii) determination of whether the phosphorylation/modification impairs G_i function. If the effects of PMA on G_i are indirect and G_i is not phosphorylated, the components preventing its inhibition of adenylyl cyclase will have to be identified. One possibility for an indirect effect is modification of the catalyst at a site that prevents the inhibitory effect of G_i.

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