Alkaline phosphatase relieves desensitization of adenylate cyclase-coupled β -adrenergic receptors in avian erythrocyte membranes

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Desensitization of adenylate cyclase-coupled β -adrenergic receptors in avian erythrocytes results in a 40–65% decrease in agonist-stimulated adenylate cyclase activity and correlates with increased phosphorylation of β -adrenergic receptors. To assess the role of phosphorylation in desensitization, membranes from isoprenaline- and dibutyryl cyclic AMP-desensitized turkey erythrocytes were incubated with alkaline phosphatase for 30 min at 37 °C, pH 8.0. In both preparations alkaline phosphatase treatment significantly decreased desensitization of agonist-stimulated adenylate cyclase activity by 40–75% ($P < 0.05$). Similar results were obtained after alkaline phosphatase treatment of membranes from isoprenaline- and dibutyryl cyclic AMP-desensitized duck erythrocytes. Moreover, alkaline phosphatase treatment of membranes from duck erythrocytes desensitized with 12-*O*-tetradecanoylphorbol 13-acetate returned agonist-stimulated adenylate cyclase activity to near control values. In all experiments, inclusion of 20 mM-sodium phosphate to inhibit alkaline phosphatase during treatment of membranes attenuated the enzyme's effect on agonist-stimulated adenylate cyclase activity. In addition, alkaline phosphatase treatment of membranes from control and isoprenaline-desensitized turkey erythrocytes increased the mobility of β -adrenergic-receptor proteins, specifically photoaffinity-labelled with [¹²⁵I]iodocyanopindolol-diazirine, on SDS/polyacrylamide-gel electrophoresis. The increased mobility of the β -adrenergic-receptor proteins after alkaline phosphatase treatment of membranes was again inhibited by 20 mM-phosphate. These results provide additional evidence for a direct role for phosphorylation in desensitization of adenylate cyclase-coupled β -adrenergic receptors in avian erythrocytes.

INTRODUCTION

Prolonged exposure of a tissue to a hormone or agonist results in the attenuated responsiveness of the tissue to a fresh challenge by the hormone or drug. This process, termed desensitization or refractoriness, appears to be a general phenomenon and affords a mechanism for the maintenance of cellular homeostasis when a tissue is continually exposed to an external stimulus. Extensive studies of tissues containing adenylate cyclase-coupled receptors have led to a proposed division in the patterns of desensitization (Harden, 1983; Sibley & Lefkowitz, 1985). Homologous desensitization is characterized by an attenuated responsiveness of adenylate cyclase to the desensitizing hormone only. Heterologous desensitization, on the other hand, results in diminished responsiveness of adenylate cyclase to all agonists, as well as to other activators such as NaF and guanine nucleotides.

The β -adrenergic-receptor–adenylate cyclase complex of avian erythrocytes provides a convenient model system in which to study heterologous desensitization. Desensitization leads to the uncoupling of the receptor from the adenylate cyclase and has been closely correlated with enhanced stoichiometric phosphorylation of the β -adrenergic receptors (Stadel *et al.*, 1983; Sibley *et al.*, 1984a). Phosphorylation of the β -adrenergic receptor

and the concomitant uncoupling of the agonist-occupied receptor from the adenylate cyclase has been observed subsequent to prolonged exposure of the erythrocytes to agonist, cyclic AMP and its analogues, or phorbol diesters such as TPA (Stadel *et al.*, 1983; Sibley *et al.*, 1984a,b; Kelleher *et al.*, 1984). Incubation of turkey erythrocyte lysates with these same agents also resulted in desensitization of the β -adrenergic-receptor–adenylate cyclase complex and increased phosphorylation of the β -adrenergic receptor, as observed in intact cells (Nambi *et al.*, 1984, 1985). These results indicate that the activation of multiple protein kinase systems is associated with the uncoupling of the β -adrenergic receptor from adenylate cyclase in these cells.

Pure β -adrenergic receptors reconstituted into phospholipid vesicles have been shown to be substrates for protein kinase preparations (Benovic *et al.*, 1985, 1986). Interestingly, the rate of phosphorylation is considerably enhanced when agonist-occupied receptors are utilized as the substrate, suggesting that agonist-induced conformational changes in β -adrenergic-receptor structure may facilitate phosphorylation. In the turkey erythrocyte system this conformational change in the β -adrenergic receptor appears to be stabilized during the desensitization/phosphorylation process and is made manifest as an apparent increase in M_r of β -adrenergic-receptor proteins after analysis by SDS/polyacrylamide-

Abbreviation used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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gel electrophoresis (Stadel *et al.*, 1986). The functional consequence of phosphorylation of the pure reconstituted β -adrenergic receptor was demonstrated by a diminished ability of agonist to promote coupling between the receptor and the stimulatory guanine nucleotide regulatory protein, G_s , as determined by GTPase assays (Benovic *et al.*, 1985, 1986).

The phosphorylated β -adrenergic receptor can be dephosphorylated by exogenous or endogenous phosphatases (Benovic *et al.*, 1985; Sibley *et al.*, 1986). However, the functional state of the dephosphorylated β -adrenergic-receptor preparations has not been reported. Although previous studies have shown an excellent correlation between phosphorylation of the β -adrenergic receptor and desensitization (Sibley *et al.*, 1984a), definitive proof that phosphorylation induces desensitization is required. The present study was undertaken to investigate whether desensitization of intact avian erythrocytes is mediated by phosphorylation, through the examination of the functional effects of alkaline phosphatase treatment on the membrane-bound β -adrenergic-receptor-adenylate cyclase complex from desensitized erythrocytes.

EXPERIMENTAL

Materials

[125 I]Iodocyanopindolol-diazirine (2200 Ci/mmol) was purchased from Amersham. Alkaline phosphatase purified from calf intestine (Molecular Biology Grade) was purchased from Calbiochem. (–)-Isoprenaline, dibutyryl cyclic AMP and TPA were obtained from Sigma. Whole turkey and duck blood was purchased from Featherdown Farms, Raleigh, NC, U.S.A.

Methods

Desensitization of avian erythrocytes. Turkey and duck erythrocytes were prepared and incubated with isoprenaline (1.0 μ M for turkey and 0.01 mM for duck), 5 mM-dibutyryl cyclic AMP, or 5 μ M-TPA (duck) for 3 h at 37 °C as previously described (Hoffman *et al.*, 1979; Sibley *et al.*, 1984b). The cells were washed three times to remove desensitizing agents. The cells were frozen and stored in liquid N₂.

Treatment of membranes with alkaline phosphatase. The cell pellets were thawed and lysed by hypo-osmotic shock in buffer containing 50 mM-Tris/HCl (pH 7.5) and 2 mM-MgCl₂. The crude membranes were then washed twice in buffer A, containing 75 mM-Tris/HCl (pH 8.0), 5 mM-MgCl₂ and 250 mM-sucrose. The membranes (~2 mg/ml) were then incubated with no additions (control), alkaline phosphatase (800 units/ml for turkey or 620 units/ml for duck), or the combination of alkaline phosphatase and 20 mM-sodium phosphate in buffer A for 30 min at 37 °C. The membranes were washed three times in buffer containing 250 mM-sucrose, 75 mM-Tris/HCl (pH 7.5), 12.5 mM-MgCl₂, 1.5 mM-EDTA and 0.5 mM-dithiothreitol. The membranes were then assayed for adenylylase activities as previously described (Hoffman *et al.*, 1979). Incubations were for 10 min at 37 °C. Proteins were determined by the method of Bradford (1976).

Photoaffinity-labelling of turkey erythrocyte β -adrenergic receptors. β -Adrenergic receptors from turkey

erythrocyte membranes were specifically labelled with [125 I]iodocyanopindolol-diazirine as previously described (Burgermeister *et al.*, 1983). Photolysis was performed for 10 min at 12 cm from a 450 W Hanovia high-pressure mercury lamp fitted with a Pyrex filter (Stadel *et al.*, 1982). [125 I]-labelled membranes were dissolved in SDS-sample buffer and analysed on SDS/12.5%-polyacrylamide gels (Laemmli, 1970). The gels were dried and exposed to Kodak XAR-5 film in the presence of intensifying screens for 1–2 weeks. Inclusion of 0.1 mM-(–)-isoprenaline or 0.01 mM-(–)-alprenolol along with [125 I]-iodocyanopindolol-diazirine during the photolysis procedures completely inhibited the incorporation of label into turkey erythrocyte-membrane proteins (Burgermeister *et al.*, 1983; results not shown).

RESULTS

Preliminary experiments were performed to optimize the conditions for incubating avian erythrocyte membranes with alkaline phosphatase. The maximal concentration of alkaline phosphatase (600–800 units/ml) that did not inhibit agonist-stimulated adenylylase activity when compared with control membranes incubated under identical conditions, but not exposed to the phosphatase, was determined (results not shown). Standard incubation conditions for the erythrocyte plasma membranes (2–4 mg/ml) were 30 min at 37 °C in the presence of 75 mM-Tris/HCl (pH 8.0)/5 mM-MgCl₂/250 mM-sucrose (see the Experimental section). The standard incubation conditions did not significantly alter isoprenaline-stimulated (7.2 ± 1.2 versus 6.3 ± 1.0 pmol of cyclic AMP/min per mg; mean $\pm$ S.E.M., $n = 7$) or NaF-stimulated (26.2 ± 3.1 versus 20.8 ± 2.6 pmol of cyclic AMP/min per mg; mean $\pm$ S.E.M., $n = 7$) adenylylase activity in control and phosphatase-treated preparations respectively. Previous studies have documented that agonists, cyclic AMP analogues and phorbol esters induce heterologous desensitization of adenylylase in avian erythrocytes (Stadel *et al.*, 1983; Sibley *et al.*, 1984b). Heterologous desensitization was determined as a small (10–15%), but significant, decrease in fluoride-stimulated adenylylase activity. Owing to the additional incubation and washings of membranes in the present experiments, statistically significant differences in fluoride-stimulated adenylylase activities between control and desensitized preparations were no longer observed, and therefore this parameter of desensitization was not studied further.

As shown in Table 1, exposure of membranes from turkey erythrocytes, desensitized by prior incubation of the cells with either isoprenaline or dibutyryl cyclic AMP, to alkaline phosphatase (800 units/ml) significantly increased the maximal isoprenaline-stimulated adenylylase activity. In the isoprenaline-desensitized preparation 43% of the desensitization was relieved, and for the dibutyryl cyclic AMP-desensitized preparation 75% of the agonist-stimulated adenylylase activity was recovered. It is noteworthy that alkaline phosphatase treatment of membranes only relieved < 50% of the desensitization induced by preincubating turkey erythrocytes with isoprenaline (see the Discussion section). The inclusion of 20 mM-phosphate with the phosphatase during membrane treatment inhibited the increase in isoprenaline-stimulated adenylylase activity observed with alkaline phosphatase alone (Table

Table 1. Effect of alkaline phosphatase on isoprenaline-stimulated adenylate cyclase activity in membranes prepared from desensitized turkey erythrocytes

Turkey erythrocytes were incubated for 3 h at 37 °C in the absence or presence of 1 μ M-isoprenaline or 5 mM-dibutyryl cyclic AMP. Membranes prepared from these cells were then treated with and without alkaline phosphatase (800 units/ml) or a combination of alkaline phosphatase and 20 mM-sodium phosphate for 30 min at 37 °C, pH 8.0. The membranes were washed and assayed for adenylate cyclase activity in the presence of 0.1 mM-isoprenaline as described in the Experimental section. Control isoprenaline-stimulated adenylate cyclase activity was 7.2 ± 1.2 pmol of cyclic AMP/min per mg; all values given are means $\pm$ S.E.M. for *n* experiments.

Pretreatment of erythrocytes	Treatment of membranes	Isoprenaline stimulation of adenylate cyclase activity (% of control)	<i>n</i>
Isoprenaline	None	40.1 ± 4.3	7
	Alkaline phosphatase	$65.7 \pm 7.2^*$	7
	Alkaline phosphatase + phosphate	$55.1 \pm 6.8^\dagger$	4
Dibutyryl cyclic AMP	None	64.2 ± 3.0	7
	Alkaline phosphatase	$91.2 \pm 3.1^*$	7
	Alkaline phosphatase + phosphate	$56.7 \pm 4.9^\ddagger$	4

* Significantly different from control (None), $P < 0.02$.

† Not significantly different from alkaline phosphatase-treated or control (None).

‡ Significantly different from alkaline phosphatase-treated, $P < 0.02$.

Table 2. Effect of alkaline phosphatase on isoprenaline-stimulated adenylate cyclase activity in membranes prepared from desensitized duck erythrocytes

Duck erythrocytes were incubated for 3 h at 37 °C in the absence or presence of 1 μ M-isoprenaline, 5 mM-dibutyryl cyclic AMP or 5 μ M-TPA. Membranes prepared from these cells were then treated with and without alkaline phosphatase (600 units/ml) or a combination of alkaline phosphatase and 20 mM-sodium phosphate for 30 min at 37 °C, pH 8.0. The membranes were washed and assayed for adenylate cyclase activity in the presence of 0.1 mM-isoprenaline as described in the Experimental section. Control isoprenaline-stimulated adenylate cyclase activity was 4.8 ± 0.7 pmol of cyclic AMP/min per mg; all values given are means $\pm$ S.E.M. for *n* experiments.

Pretreatment of erythrocytes	Treatment of membranes	Isoprenaline stimulation of adenylate cyclase activity (% of control)	<i>n</i>
Isoprenaline	None	43.5 ± 2.4	7
	Alkaline phosphatase	$67.6 \pm 8.6^*$	7
	Alkaline phosphatase + phosphate	$52.8 \pm 4.9^\dagger$	5
Dibutyryl cyclic AMP	None	51.1 ± 3.7	7
	Alkaline phosphatase	$80.7 \pm 7.2^*$	7
	Alkaline phosphatase + phosphate	$49.2 \pm 5.3^\dagger$	5
TPA	None	62.3 ± 4.6	7
	Alkaline phosphatase	$86.0 \pm 6.7^*$	7
	Alkaline phosphatase + phosphate	$60.1 \pm 4.7^\dagger$	5

* Significantly different from control (None), $P < 0.05$.

† Significantly different from alkaline phosphatase-treated, $P < 0.05$.

1). This concentration of phosphate has been shown to inhibit alkaline phosphatase activity (Fernley & Walker, 1967; Stinson & Seargeant, 1981). In four experiments a consistent pattern was observed in which alkaline phosphatase relieved desensitization as determined by agonist-stimulated adenylate cyclase activity, and 20 mM-phosphate inhibited this result. The effect of phosphate on that of the phosphatase was statistically significant ($P < 0.02$) when membranes from dibutyryl cyclic AMP-

desensitized erythrocytes were examined; however, the effect on membranes from isoprenaline-desensitized cells, although qualitatively similar, was not statistically significant. These results support the notion that the increased isoprenaline-stimulated adenylate cyclase activity after treatment of membranes with alkaline phosphatase was indeed due to dephosphorylation.

The effect of alkaline phosphatase on desensitization was further investigated in duck erythrocyte membranes.

Table 2 demonstrates that under standard incubation conditions alkaline phosphatase (620 units/ml) relieved the attenuated agonist-stimulated adenylate cyclase activity in membranes from duck erythrocytes pre-incubated with isoprenaline or dibutyryl cyclic AMP. In addition, desensitization induced by the phorbol diester TPA was also decreased by treating membranes with alkaline phosphatase. In all three desensitized preparations (agonist-, dibutyryl cyclic AMP-, and TPA-desensitized) 20 mM-phosphate significantly inhibited the effect of alkaline phosphatase on isoprenaline-stimulated adenylate cyclase activity. The results obtained in the two avian erythrocyte systems were very similar. However, phorbol diester-induced desensitization in turkey erythrocytes was not consistently observed, and therefore was not pursued in that cell type. Although previous investigators have reported that agonist occupancy enhanced the dephosphorylation of pure exogenously phosphorylated β -adrenergic receptors by purified phosphoprotein phosphatase (Benovic *et al.*, 1985), the addition of isoprenaline (0.1 mM) or (-)-alprenolol (0.01 mM) along with the alkaline phosphatase had no detectable enhancing effect on resensitization of the avian erythrocyte adenylate cyclase (results not shown).

Desensitization of turkey erythrocyte adenylate cyclase has been correlated with increased phosphorylation of the β -adrenergic receptor and decreased mobility of the receptor protein on SDS/polyacrylamide-gel electrophoresis (Stadel *et al.*, 1982, 1983; Sibley *et al.*, 1984a). A mobility shift on SDS/polyacrylamide gels for β -adrenergic receptors from desensitized duck erythrocytes is less apparent. Since alkaline phosphatase treatment of membranes from desensitized cells increased agonist-stimulated adenylate cyclase activity, it was decided to determine if the phosphatase exerted a measurable effect on the mobility of the β -adrenergic receptors. After treatment of membranes from turkey erythrocytes, desensitized by prior exposure of intact cells to isoprenaline, with alkaline phosphatase or the combination of alkaline phosphatase and 20 mM-phosphate, the membranes were washed and subsequently labelled with the specific photoaffinity label [125 I]iodocyanopindolol-diazirine (Burgermeister *et al.*, 1983). The [125 I]-labelled β -adrenergic-receptor preparations were then analysed by SDS/polyacrylamide-gel electrophoresis and an autoradiogram of such a gel is shown in Fig. 1. As previously observed (Stadel *et al.*, 1982), agonist-promoted desensitization decreased the mobility of the labelled β -adrenergic-receptor protein compared with control preparations. Treatment of membranes with alkaline phosphatase increased the mobility of both control and desensitized β -adrenergic receptors. After alkaline phosphatase treatment, the β -adrenergic-receptor preparations appeared as broad bands, suggesting heterogeneity, i.e. incomplete dephosphorylation. The altered mobility of both control and desensitized β -adrenergic receptors after alkaline phosphatase treatment was inhibited when 20 mM-phosphate was included along with the enzyme during the membrane treatment. The reason for variable labelling of the β -adrenergic-receptor proteins after treatment of membranes with alkaline phosphate or with the combination of the phosphatase and phosphate is not known. However, the effect of alkaline phosphate on both isoprenaline-stimulated adenylate cyclase activity and β -adrenergic

Fig. 1. SDS/polyacrylamide-gel electrophoresis of [125 I]iodocyanopindolol-diazirine-labelled β -adrenergic receptors from membranes of control and isoprenaline-desensitized turkey erythrocytes treated with alkaline phosphatase in the absence and presence of phosphate

Turkey erythrocytes were incubated for 3 h at 37 °C in the presence (D) or absence (C) of 1 μ M-isoprenaline. Membranes prepared from these cells were treated without or with alkaline phosphatase (800 units/ml) or a combination of alkaline phosphatase and 20 mM-sodium phosphate as indicated. The membranes were then washed and photoaffinity-labelled with [125 I]iodocyanopindolol-diazirine as described in the Experimental section. The [125 I]-labelled membranes were dissolved in SDS sample buffer and analysed on a SDS/12.5%-polyacrylamide gel. The gel was dried and exposed to Kodak XAR-5 film for 7 days. M_r standards were albumin (M_r 67000), ovalbumin (M_r 43000) and carbonic anhydrase (M_r 30000). The experiment was repeated with similar results.

receptor mobility on SDS/polyacrylamide gels was inhibited by phosphate, suggesting that receptor phosphorylation contributes to the uncoupling of the β -adrenergic receptor from the adenylate cyclase during desensitization in this cell type.

DISCUSSION

The results of the present study indicate a functional role for phosphorylation during desensitization of adenylate cyclase-coupled β -adrenergic receptors in intact avian erythrocytes. These data are consistent with previous observations using pure reconstituted β -adrenergic receptors and purified protein kinase and phosphoprotein phosphatase preparations (Benovic *et al.*, 1985, 1986). Our results extend these previous studies in two ways. First, we have examined the functional significance of phosphorylation after exposure of intact avian erythrocytes to several agents that induce desensitization of the β -adrenergic-receptor-adenylate cyclase complex. Second, we show that alkaline phos-

phatase treatment of membranes from desensitized intact cells promotes enhanced coupling of the β -adrenergic receptor to the effector adenylate cyclase, partially resensitizing the transmembrane signalling system. Similar results have been reported with membranes from pig ovarian follicles (Hunzicker-Dunn *et al.*, 1979). Treatment of follicular membranes, desensitized to luteinizing hormone, with a partially purified phosphoprotein phosphatase preparation resensitized the luteinizing-hormone-receptor-adenylate cyclase complex.

Alkaline phosphatase treatment of membranes from both control and isoprenaline-desensitized turkey erythrocytes increased the mobility of the β -adrenergic receptor proteins on SDS/polyacrylamide gels. β -Adrenergic receptors from control turkey erythrocytes have been shown to be phosphoproteins (Stadel *et al.*, 1983; Sibley *et al.*, 1984a) containing nearly 1 mol of phosphate/mol of receptor; desensitization promotes a stoichiometric increase in the phosphorylation of the turkey erythrocyte β -adrenergic receptors. The increased mobility of control β -adrenergic receptors on SDS/polyacrylamide gels may represent the totally dephosphorylated state of the receptor after phosphatase treatment. This result suggests that the 'basal' phosphate of the receptor does not appear to be required for ligand recognition or coupling to adenylate cyclase. The broad bands observed for both the control and desensitized β -adrenergic receptors after treatment with alkaline phosphatase suggests heterogeneity in the dephosphorylated forms of the receptors in this preparation. The effect of alkaline phosphatase on β -adrenergic-receptor mobility on SDS/polyacrylamide gels was inhibited by phosphate, similar to the results on the resensitization of the adenylate cyclase, suggesting both the altered receptor mobility and resensitization are mediated by dephosphorylation and are not due to contaminating proteinase activities.

Relatively high concentrations of alkaline phosphatase were used in the treatment of the erythrocyte membranes. Alkaline phosphatase did not dephosphorylate pure reconstituted β -adrenergic receptors (Benovic *et al.*, 1985). It is possible that, by using a highly purified preparation of alkaline phosphatase, we were able to employ sufficiently high concentrations to promote dephosphorylation. The necessity of using such high concentrations of the phosphatase may reflect the possibility that the phosphorylation sites in membranes from desensitized cells are not readily accessible to or well recognized by alkaline phosphatase. In addition, the incubation conditions were optimized to maintain the integrity of the adenylate cyclase complex and were not optimal for alkaline phosphatase activity.

In the avian erythrocyte systems alkaline phosphatase treatment relieved isoprenaline-induced desensitization by 40–50%, whereas similar treatment decreased dibutyl cyclic AMP- and TPA-induced desensitization to a greater extent, and in some experiments the resensitization appeared to be nearly complete. Several factors may explain these observations. Previous studies suggest that agonist-induced desensitization of turkey erythrocytes may involve a two-step mechanism (Stadel *et al.*, 1987). Cyclic AMP-dependent protein kinase activity can only account for ~50% of maximal desensitization of the adenylate cyclase activity observed in the presence of agonist and ~50% of the agonist-promoted increase in

phosphorylation of the β -adrenergic receptor (Nambi *et al.*, 1985). The additional desensitization/phosphorylation observed after agonist pretreatment of cells may involve an additional protein kinase. The additional kinase is unlikely to be protein kinase C, as alkaline phosphatase treatment of membranes relieved TPA-induced desensitization. However, a second protein kinase, presumably similar to the recently described β -adrenergic-receptor kinase (Benovic *et al.*, 1986), could be involved. It may be that alkaline phosphatase does not remove the unique phosphate added to the β -adrenergic receptor in the presence of agonist (Stadel *et al.*, 1987). A second possibility is that agonist-induced desensitization of intact erythrocytes promotes the phosphorylation of an additional membrane component regulating adenylate cyclase activity, e.g. G_s or the catalytic component of adenylate cyclase. This possibility could help to explain the heterologous nature of desensitization in these cells after prolonged exposure to catecholamines (Hoffman *et al.*, 1979). Again, this additional component would appear to be a poor substrate for alkaline phosphatase. Finally, our data do not rule out the possibility that in whole cells a second mechanism, in addition to phosphorylation, may be involved in the agonist-induced desensitization process, and therefore would be resistant to alkaline phosphatase treatment.

In summary, biochemical properties of the β -adrenergic receptor and agonist-stimulated adenylate cyclase activity in membranes from desensitized erythrocytes were modulated in parallel by alkaline phosphatase. Our results provide evidence that desensitization-dependent phosphorylation functionally inhibits coupling between the β -adrenergic receptor and adenylate cyclase.

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