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β-Adrenergic Desensitization Reduces the Sensitivity of Adenylate Cyclase for Magnesium in Permeabilized Lymphocytes

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SUMMARY

Magnesium modulates hormone-sensitive adenylate cyclase activation. In the present studies, we have examined the magnesium requirement of β-adrenergic-stimulated adenylate cyclase activity in permeabilized human lymphocytes. Following isoproterenol pretreatment, under conditions that lead to homologous β-adrenergic desensitization, the EC_{50} of magnesium for β-adrenergic-stimulated adenylate cyclase activity was significantly increased [control; 1.99 (+0.81/−0.57) mM; desensitized; 3.82 (+0.31/−0.29) mM]. Further, when assays were performed at high Mg^{2+} concentrations following agonist pretreatment, we detected only small and inconsistant reductions in β-adrenergic-stimulated adenylate cyclase activity and in β-adrenergic cAMP-dependent protein kinase activity. In contrast, the detection of agonist-induced β-adrenergic receptor sequestration and alterations in receptor affinity for agonists was not qualitatively affected by changes in magnesium concentrations. The data demonstrate that the functional consequences of β-adrenergic desensitization may be obscured at high magnesium concentrations. Furthermore, in this system desensitization may be viewed as a β-receptor-specific reduction in magnesium sensitivity.

Receptor-mediated stimulation and inhibition of adenylate cyclase activity is modulated by several monovalent and divalent cations (1). For receptors linked to activation of adenylate cyclase activity, magnesium modulates the receptor·G protein·catalytic interactions at several sites (2–4). At the catalytic site, magnesium acts as a cofactor with ATP. At the level of the G protein, there is a magnesium requirement for GTPase activity as well as for hormone-stimulated G_s activation and GTP binding. Also, magnesium is required for detection of the so called "high affinity state of the receptor for agonist."

Activation of receptors linked to adenylate cyclase activity may also modulate magnesium effect. In the hepatocyte, glucagon receptor occupancy may result in a reduction in the EC_{50} of magnesium for adenylate cyclase activity, probably via increased G_s activation (4). It has also been suggested that hormones that act via inhibition of adenylate cyclase activity may do so by increasing the EC_{50} of magnesium for receptor-stimulated adenylate cyclase activity (5). More recently, it has been suggested that the ability to detect desensitization of the β-adrenergic receptor may be Mg^{2+}-dependent. In the S_{49} lymphoma cell, Clark *et al.* (6) reported that the agonist-induced reduction of both β-adrenergic adenylate cyclase activity and β-receptor affinity for agonists was unmasked only at submillimolar Mg^{2+} concentration. In that study, β-adrenergic desensitization was characterized as "heterologous" based on a concurrent reduction in both isoproterenol and PGE_1-stimulated activity as well as on the absence of apparent β-receptor sequestration (6, 7).

The present studies were performed to further explore the effects of Mg^{2+} on the human lymphocyte β-adrenergic receptor, a model in which a homologous pattern of desensitization is predominant [reflected by a β-adrenergic agonist-specific reduction in adenylate cyclase activity without alterations in the effect of other stimulators and with concurrent β-receptor sequestration (8–10)].

The effects of magnesium have generally been studied in broken cell preparations. In contrast, we have utilized a permeabilized cell preparation in order to minimize perturbation of the system. Our data demonstrate that the ability to detect functional homologous desensitization (in terms of reduced β-adrenergic-mediated adenylate cyclase and cAMP-dependent protein kinase activities) is attenuated at high concentrations of magnesium. Furthermore, homologous desensitization of the human lymphocyte β-adrenergic receptor is associated with an apparent increase in the EC_{50} of magnesium for hormonal stimulation of adenylate cyclase activity. However, in contrast to previous studies in the S_{49} cell model of heterologous desensitization, the ability to detect agonist-induced alterations in

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ABBREVIATIONS: G protein, guanine nucleotide regulatory protein; PGE_1, prostaglandin E_1; G_s, stimulatory G protein; IPIN, [^{125}I]iodopindolol; Gpp(NH)p, guanylimidodiphosphate; K_L, dissociation constant for the low affinity state; K_H, dissociation constant for the high affinity state; $\%R_H$, proportion of receptors binding agonist with a high affinity. HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

receptor-hormone sensitivity and receptor sequestration in the human lymphocyte is not Mg^{2+} dependent.

Materials and Methods

Permeabilized cell preparation. The protocol for cell permeabilization was adapted from the method of Brooker and Pedone (11). EDTA-anticoagulated blood (50–100 ml) was drawn from drug-free normotensive subjects between the ages of 18 and 35. Mononuclear leukocytes were isolated according to the method of Boyum (12) but were maintained at 4° during isolation and permeabilization to inhibit *ex vivo* alterations in lymphocyte β-receptor properties (13). After separation on a Ficoll-Hypaque gradient, mononuclear cells were harvested as previously described (14), resuspended in Hanks' balanced salt solution (without Ca^{2+} and Mg^{2+}), centrifuged for 10 min at 400 × *g*, and resuspended in Hanks' balanced salt solution (pH 7.4 at 4°) with 33 mM HEPES, 0.5 mM EDTA (Buffer A) and varying concentrations of magnesium sulfate.[1] Cells were permeabilized with digitonin (Sigma Chemical Co., St. Louis, MO) that had been prepared by dissolving in boiling water (6 g/60 ml), cooling overnight at room temperature, filtering to remove insoluble material, and lyophilizing. A final digitonin concentration of 1 mg/100 ml was used for studies of adenylate cyclase activity, cAMP-dependent protein kinase activity, and radioligand binding studies involving determination of the proportion of CGP-12177-accessible cells. A final digitonin concentration of 7.5 mg/100 ml was used for radioligand binding studies relating to isoproterenol competition binding and guanine nucleotide sensitivity. These digitonin concentrations were based on preliminary studies in which it was determined that adenylate cyclase activity was markedly decreased at digitonin concentrations greater than 3 mg/100 ml. Treatment with 1 mg/100 ml digitonin resulted in optimal detection of adenylate cyclase activity. However, the ability of guanine nucleotides to shift agonist competition curves was significantly diminished at digitonin concentrations of 1 mg/100 ml and optimal guanine nucleotide sensitivity was achieved at 7.5 mg/100 ml digitonin. In contrast, agonist-induced alterations in CGP-12177-accessible labeling were comparable at 1 or 7.5 mg/100 ml digitonin. Cells were incubated for 15 min at 4°, washed at 400 × *g* for 10 min at 4°, resuspended in Buffer A, washed again, and resuspended for radioligand binding studies or assays of adenylate cyclase activity as described below. After treatment with either 1 or 7.5 mg/100 ml digitonin, greater than 90% of cells still excluded trypan blue.

Assays of adenylate cyclase activity. Assays of adenylate cyclase activity were performed on permeabilized cell preparations according to our previously published methods (14) modified after Salomon *et al.* (15). Maximal isoproterenol-stimulated adenylate cyclase activity was expressed as the difference between activity in the presence of isoproterenol (100 μM) and GTP (100 μM) and activity in the presence of GTP alone. Maximal PGE_1 (100 μM)-stimulated activity was assayed similarly. Adenylate cyclase activity was linear with time and cell number over the ranges used in these studies. Isoproterenol stimulation of adenylate cyclase activity was significantly higher in permeabilized cells than with our previous crude broken cell preparations (14) (permeabilized cells, 211 ± 15% of GTP-stimulated activity; broken cells, 152 ± 9%, n = 5).

Assays of cAMP-dependent protein kinase activation. Assays of cAMP-dependent protein kinase activity were performed in permeabilized cells according to modifications of the methods of Roskowski (16). Permeabilized cells in Buffer A and varying concentrations of $MgSO_4$ were incubated for 12 min at 30° with 1 mM Kemptide (Sigma), 0.5 mM isobutylmethylxanthine, 1 μg/ml of bovine serum albumin, 0.5 mM ascorbic acid, 0.8 mM ATP, 1–2 μCi of [α-^{32}P]ATP, and 100 μM GTP, in a final volume of 100 μl. Reactions were terminated by spotting aliquots (80 μl) on 2 × 3 cm phosphocellulose strips (Whatman P81) and immersing them in 75 mM phosphoric acid. The strips were swirled gently for 2 min, the phosphoric acid was decanted, and the strips were washed twice more as above. Radioactivity was measured by liquid scintillation counting (Beckman LS 3801). Background was determined by blanks incubated in the absence of Kemptide, cells, or [^{32}P]ATP alone and generally accounted for less than 15% of basal activity. Protein kinase activity was linear with time up to at least 20 min. Maximal levels of stimulated activity were determined in the presence of PGE_1 (100 μM), isoproterenol (100 μM), or cAMP (100 μM). Hormonal activity was expressed as the ratio of hormone-stimulated activity to cAMP-stimulated activity. The EC_{50} of isoproterenol for protein kinase activation at Mg^{2+} = 1.5 mM was comparable to that for adenylate cyclase activity [EC_{50} (protein kinase) = 132 nM (+60/−37), three experiments; EC_{50} (adenylate cyclase) = 218 nM (+9/−9), 11 experiments].

Radioligand binding studies. Radioligand binding studies were performed in permeabilized lymphocytes according to our previously described methods (17) with the use of IPIN (2.2 Ci/μmol; New England Nuclear, Boston, MA). Assays were performed at 25° for 90 min, by which time steady state was achieved at all radioligand concentrations used.

Saturation binding curves were used to determine receptor density (B_{max}) and receptor affinity for the radioligand (K_D). IPIN labeling in the presence of propranolol (1 μM) was defined as nonspecific binding. The proportion of specific binding blocked by the hydrophilic ligand CGP-12177 (30 nM) was used as an index of "nonsequestered" β-receptor sites, as previously described (9).

Receptor affinity for agonists was determined from radioligand-agonist competition curves using permeabilized cells in the presence and absence of Gpp(NH)p, as previously described (14). As compared with our previous broken cell preparation, β-receptor affinity for isoproterenol in the absence of Gpp(NH)p is increased up to 10-fold in the permeabilized cell preparation (IC_{50} for isoproterenol in broken cells previously reported as 240 ± 20 nM (14) and 184 ± 24 (18) versus IC_{50} of isoproterenol of 17 ± 0.2 nM in permeabilized cells at a final Mg^{2+} concentration of 10 mM).

Data analyses. Radioligand binding data were analyzed as previously described, using a computerized curve-fitting program (LIGAND) (14). Saturation binding curves were analyzed for receptor density and receptor affinity for IPIN. Agonist competition curves in the absence of guanine nucleotides were analyzed according to both a one- and a two-affinity site model of agonists. The two-affinity site model was accepted only if it significantly improved the fit of the data over the one-site model. Overall IC_{50} for isoproterenol from agonist-radioligand competition curves and EC_{50} for dose-response curves for adenylate cyclase activity were determined by computerized nonlinear sigmoid curve-fitting of the data (GraphPad ISI).

All expressions of potency (EC_{50}, K_i, and K_D) are expressed as the geometric mean ± standard error. All other parameters are expressed as arithmetic mean ± standard error.

Desensitization protocol. Agonist-induced alterations in lymphocyte β-receptors were examined using an *in vitro* desensitization protocol, as previously described (19). One half of each EDTA-anticoagulated whole blood sample was incubated with isoproterenol (10 μM for 10 min), followed by cell separation at 4° and studies of adenylate cyclase activity and radioligand binding as described above. In broken cell preparations with a final Mg^{2+} concentration of 9.7 mM, this treatment is associated with a greater than 50% reduction in β-receptor-mediated adenylate cyclase activity without change in PGE_1-, sodium fluoride-, and Gpp(NH)p-stimulated activities, consistent with a homologous pattern of desensitization (8, 9). Under these conditions, no down-regulation occurs (no change in B_{max} for β-adrenergic-specific (IPIN) binding); however, apparent sequestration of approximately 50% of the receptor population can be demonstrated (9, 19).

Results

Mg^{2+} EC_{50} for adenylate cyclase activity. When adenylate cyclase activity is expressed as a function of [Mg^{2+}], an

[1] The Mg^{2+} concentrations stated here represent values of $MgSO_4$ added in excess over ATP and EDTA and, hence, are estimates of free intracellular Mg^{2+}.

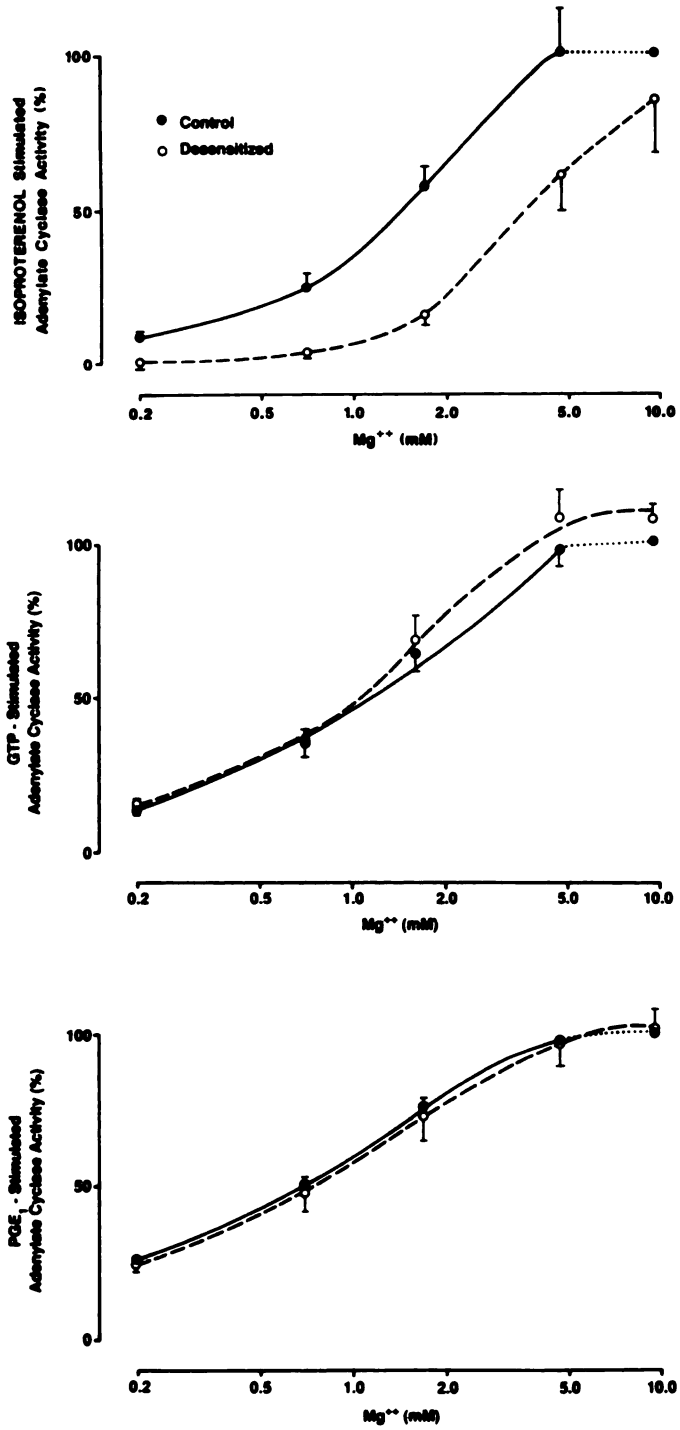

Fig. 1. Alterations in EC_{50} of Mg^{2+} for adenylate cyclase activity in control (●) and desensitized cells (○). *Upper*, Alterations in isoproterenol-stimulated adenylate cyclase activity. The mean ± standard error from triplicate determinations from five studies are shown. In each experiment, isoproterenol-stimulated adenylate cyclase activity in control cells at the highest Mg^{2+} concentration was set at 100% and represented an increase in cAMP production of 38.5 ± 9.4 pmol/10 min/10^6 cells over GTP-stimulated levels. Mg^{2+} EC_{50} and maximal activity were determined for control and desensitized cells by computerized curve-fitting analysis (GraphPad program, Nonlinear Regression-Sigmoid Curve Subroutine). *Middle*, Alterations in GTP-stimulated activity. The mean ± standard error from triplicate determinations from 11 studies are shown. GTP-stimulated adenylate cyclase activity in control cells at the highest Mg^{2+} concentration was set at 100 and represented 15.2 ± 2.1 pmol of cAMP/10 min/10^6 cells (and 29.2 ± 5.9 pmol of cAMP/min/mg of protein). EC_{50} was determined as above. *Lower*, Alterations in PGE_1-stimulated activity. The mean ± standard error from triplicate determinations from six studies are shown. In each experiment PGE_1-stimulated adenylate cyclase activity in control cells at the highest Mg^{2+} concentration was set at 100% and represented 45.4 ± 9.5 pmol of cAMP/10 min/10^6 cells (and 82.6 ± 25.3 pmol of cAMP/min/mg of protein). EC_{50} for Mg^{2+} was determined as above.

EC_{50} of Mg^{2+} can be determined. In the permeabilized lymphocyte preparation, we determined the EC_{50} of Mg^{2+} to be in the range of 0.7 to 2.0 mM. Specifically, the EC_{50} of Mg^{2+} for basal adenylate cyclase activity (in the presence of GTP alone) was 1.26 mM (+0.26/−0.24); the EC_{50} of Mg^{2+} for isoproterenol-stimulated adenylate cyclase activity was 1.99 mM (+0.81/−0.57); and the EC_{50} of Mg^{2+} for PGE_1-stimulated adenylate cyclase activity was 0.97 mM (+0.25/−0.20).

Effect of isoproterenol-pretreatment on Mg^{2+} EC_{50}. The effect of β-adrenergic agonist pretreatment on the Mg^{2+} EC_{50} was determined for (a) basal adenylate cyclase activity (with GTP alone), (b) PGE_1-stimulated adenylate cyclase activity, and (c) isoproterenol-stimulated adenylate cyclase activity. As illustrated in Fig. 1, the relationship between $[Mg^{2+}]$ and PGE_1 and GTP-stimulated adenylate cyclase activity was unaltered by agonist pretreatment, i.e., the Mg^{2+} EC_{50} values for PGE_1- and GTP-stimulated adenylate cyclase activities were essentially unchanged (Table 1). In contrast, the EC_{50} of Mg^{2+} for isoproterenol-stimulated adenylate cyclase activity was increased approximately 2-fold with agonist pretreatment, from 1.99 mM (+0.81/−0.57) to 3.82 mM (+0.31/−0.29) (Fig. 1).

An alternate approach in expressing these data is illustrated in Fig. 2. From this perspective, it is apparent that the ability to detect β-adrenergic desensitization is dependent on Mg^{2+} concentration. When adenylate cyclase activity was assayed at lower Mg^{2+} concentrations, agonist exposure resulted in almost complete attenuation of detectable isoproterenol-stimulated adenylate cyclase activity. However, at higher Mg^{2+} concentrations, only a small and inconsistent reduction in maximal β-adrenergic-stimulated adenylate cyclase activity could be detected.

We next determined whether, at high Mg^{2+} levels, an agonist-induced reduction in the potency of isoproterenol (EC_{50} of isoproterenol) for adenylate cyclase activity might now be detected under conditions in which a reduction in maximal isoproterenol-stimulated adenylate cyclase activity was no longer apparent. However, after agonist pretreatment, no changes in the EC_{50} of isoproterenol for adenylate cyclase activity were detectable at $[Mg^{2+}]$ = 9.7 mM [EC_{50} of isoproterenol (control), 190 nM (+20/−19); EC_{50} of isoproterenol (treated), 159 nM, (+2/−1), three experiments] (Fig. 3). Therefore, no changes in either EC_{50} or V_{max} for isoproterenol can be detected at the highest Mg^{2+} concentration.

Effect of isoproterenol pretreatment on cAMP-dependent protein kinase activity. We next determined whether the ability to detect β-adrenergic desensitization at the level of cAMP-dependent protein kinase was similarly Mg^{2+} dependent. An agonist-induced reduction in maximal isoproterenol-stimulated activity could be demonstrated at a low Mg^{2+} concentration (0.5 mM). However, only a small, inconsistant reduction was seen at higher $[Mg^{2+}]$ (Fig. 4). PGE_1-stimulated activity was not significantly altered after isoproterenol pretreatment at either Mg^{2+} concentration (Fig. 4). Further, protein kinase activity in the presence of GTP only (100 μM) was

TABLE 1

Mg^{2+} EC_{50} for adenylate cyclase activity: effects of isoproterenol pretreatment

Data are presented as mean ± geometric error of the mean as derived from nonlinear curve fitting estimates.

	EC_{50}	
	GTP-stimulated activity ($n = 11$)	PGE_1-stimulated activity ($n = 6$)
	mM	
Control	1.54 (+0.30/−0.24)	0.74 (+.09/−.08)
Isoproterenol-treated	1.91 (+0.32/−0.28)	0.87 (+.12/−.10)

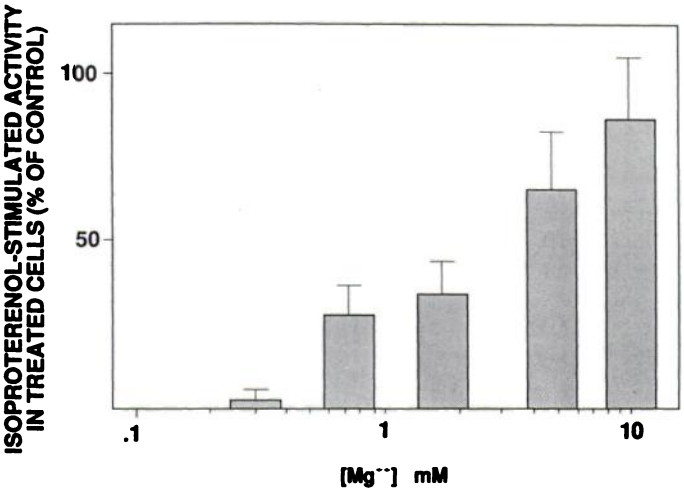

Fig. 2. The effect of increasing Mg^{2+} concentration on the ability to detect β-adrenergic agonist-induced desensitization. Maximal isoproterenol-stimulated adenylate cyclase activity in agonist-treated cells was expressed as a percentage of the activity in control cells at each of the five Mg^{2+} concentrations studied. Data represent the mean ± standard error of triplicate determinations from each of five studies.

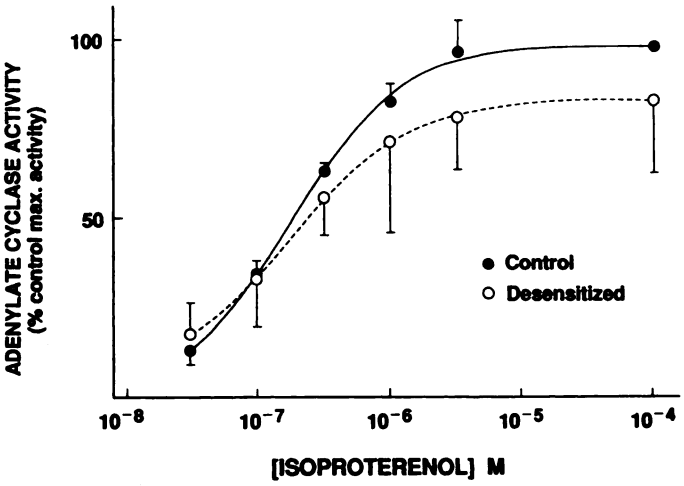

Fig. 3. Isoproterenol-stimulated adenylate cyclase activity in control (●) and desensitized cells (○), high [Mg^{2+}] (9.7 mM). In each experiment, the increase in adenylate cyclase activity stimulated by 100 μM isoproterenol in control cells was set at 100% and the extent of stimulation at other isoproterenol concentrations in control cells and at all concentrations in agonist-pretreated cells was expressed relatively. Data represent the mean ± standard error of triplicate determinations in each of three separate experiments.

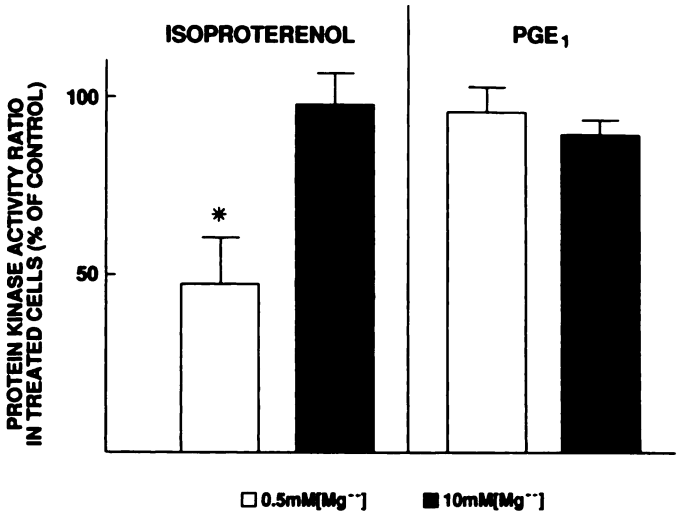

Fig. 4. Alterations in cAMP-dependent protein kinase activity ratios following isoproterenol pretreatment, low (0.5 mM) versus high (10 mM) [Mg^{2+}]. Data represent the mean ± standard error from five separate experiments of isoproterenol- and PGE_1-stimulated protein kinase activity ratios from isoproterenol-pretreated cells. The data are expressed relative to control activity ratios, which were isoproterenol (low Mg^{2+}) 0.34 ± 0.09; isoproterenol (high Mg^{2+}), 0.48 ± 0.06; PGE_1 (low Mg^{2+}), 0.44 ± 0.11; PGE_1 (high Mg^{2+}), 0.57 ± 0.12. *$p < 0.05$ versus control levels by Student's t test.

not altered with agonist pretreatment (low Mg^{2+}: control, 112 ± 39 pmol/min/10^6 cells; isoproterenol-treated, 115 ± 29; high Mg^{2+}: control, 63 ± 12; isoproterenol-treated, 57 ± 11; mean ± standard error from triplicate determinations from seven separate studies).

As noted above, desensitization of β-adrenergic response was detected (at low Mg^{2+}) in terms of a reduction in maximal protein kinase activation. It might have been hypothesized that a reduction in sensitivity for isoproterenol would have been a more likely consequence of desensitization. However, in this model the coupling ratio of the EC_{50} values of isoproterenol for adenylate cyclase/protein kinase activation is relatively low (<2). Thus, under these circumstances a reduction in maximal response would in fact be the expected response.

Effect of magnesium on agonist-induced sequestration. Might higher magnesium concentrations in the assay also attenuate the ability to detect agonist-induced sequestration of the β-receptor? To answer this question, we assessed the ability of CGP-12177 to compete for IPIN binding in permeabilized lymphocytes. CGP-12177 is a hydrophilic β-adrenergic antagonist that has been demonstrated to bind selectively to non-sequestered receptors (20–22). In intact cells, CGP-12177 (30 nM) competes for greater than 90% of β-receptor-specific IPIN binding sites under control conditions but for less than 50% of sites after agonist exposure, consistent with sequestration of a significant proportion of the receptor population (17, 19). Under control conditions, the proportion of CGP-12177-accessible binding sites in permeabilized cells was greater than 90%. As illustrated in Table 2, agonist-induced sequestration in permeabilized cells was similar to that previously reported in intact cells. Further, higher Mg^{2+} concentrations did not alter the ability to detect this process. Receptor density (B_{max}) and receptor affinity for the radioligand antagonist IPIN were similarly unaffected by the magnesium concentration in the assay (Table 2).

TABLE 2

Agonist-induced alterations in radioligand binding saturation curves

The data are the mean ± standard error of parameters derived from saturation binding curves performed on three occasions. The K_D of IPIN is expressed as the mean ± geometric error of the mean.

	0.5 mM Mg^{2+}		10 mM Mg^{2+}	
	Control	Desensitized	Control	Desensitized
K_D (pM)	38 (+43/−34)	43 (+2/−2)	40 (+9/−8)	26 (+8/−7)
B_{max} (sites/cell)	601 ± 44	525 ± 29	605 ± 93	584 ± 22
% CGP-12177 Accessible Sites	92 ± 1	44 ± 1	92 ± 0	40 ± 3

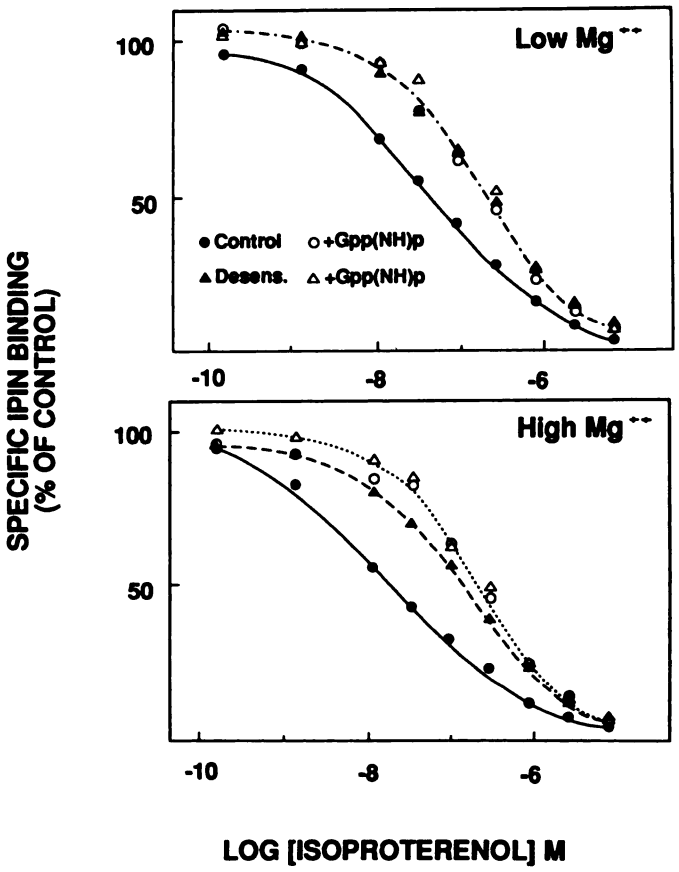

Fig. 5. Alterations in receptor affinity for agonist with isoproterenol pretreatment at low (0.5 mM) Mg^{2+} (*upper*) and high (10 mM) Mg^{2+} (*lower*). Data represent the mean of triplicate determinations from each of five studies performed under identical conditions.

Effect of magnesium on the alteration in receptor affinity for agonists with isoproterenol pretreatment. Because higher magnesium levels masked the reduction in β-adrenergic mediated adenylate cyclase activity normally seen with agonist pretreatment, one might anticipate that the ability to detect a reduction in receptor affinity for agonist would also be blunted. In untreated cells, receptor affinity for isoproterenol was increased when assayed in the presence of higher magnesium concentrations (10 mM; Table 3; Fig. 5). This was characterized as a higher ratio of dissociation constants for the high and low affinity states of the receptor for the agonists (K_L/K_H ratio; see Table 3). However, agonist pretreatment reduced receptor affinity for isoproterenol when assayed at either high (10 mM) or low (0.5 mM) Mg^{2+} concentrations. Additionally, there were comparable reductions in the proportion of high affinity state receptors ($\%R_H$) and K_L/K_H ratio (Table 3).

Discussion

The present studies demonstrate that, in the permeabilized lymphocyte preparation under conditions leading to homologous desensitization, there is an agonist-induced/β-receptor-specific shift in the EC_{50} of Mg^{2+} for adenylate cyclase activation. Further, when assayed at high Mg^{2+} concentrations, the ability to detect an agonist-induced reduction in both β-adrenergic adenylate cyclase activity and β-adrenergic cAMP-dependent protein kinase activity is attenuated. In contrast, the ability to detect both the reduction in receptor affinity for agonist and β-receptor sequestration following isoproterenol exposure was not affected by assaying at higher Mg^{2+} concentration.

The implications of these studies can be appreciated on both technical and conceptual levels. On a technical level, these studies suggest the permeabilized lymphocyte may be very useful in the study of the human β-adrenergic receptor complex. At maximal Mg^{2+} concentrations, the extent of isoproterenol-stimulated adenylate cyclase activity in this preparation is similar to that in intact lymphocyte cAMP accumulation assays (23–27) and is approximately 2-fold higher than the activity we have previously reported in broken cell preparations.[2] The permeabilized lymphocyte can also be used for assessment of guanine nucleotide-sensitive receptor affinity for agonist as well as CGP-12177 accessibility. Previously, these assays could only be performed in broken cell and intact cell preparations, respectively. Additionally, these studies demonstrate the utility of this model for the determination of cAMP-dependent protein kinase activity ratios. Thus, the permeabilized cell preparation provides a useful approach for studying the process of desensitization at multiple levels in the hormone receptor-G protein-adenylate cyclase-cAMP-dependent protein kinase pathway.

In radioligand binding studies, CGP-12177 is still able to differentiate a sequestered β-receptor population in lymphocytes permeable to small hydrophilic molecules (e.g., permeable to GTP, ATP, Mg^{2+}). This observation offers an interesting insight into the process of lymphocyte β-adrenergic desensitization. In intact cell studies, the agonist-induced reduction in CGP-12177 accessibility has been generally related to its comparative inability to cross the cell membrane. However, the current studies suggest that sequestered receptors may still be inaccessible to CGP-12177, even under conditions where the ligand can cross the cell membrane. Perhaps the sequestered receptors are within a lipophilic compartment, either in an intracellular compartment or, perhaps more likely, within the plasma membrane as suggested by Mahan *et al.* (28) and Strader *et al.* (29). However, although the morphological correlate of the sequestered state remains unclear, the data suggest that the sequestered receptors do not reside in a hydrophilic cytoplasmic compartment.

Classically, desensitization of β-adrenergic receptor systems has been considered as either homologous or heterologous based on such characteristics as 1) whether attenuation of response is β-adrenergic specific or generalized to other receptor stimu-

[2] It should, however, also be noted that basal adenylate cyclase activity seems to be considerably higher in the digitonin-treated lymphocytes than that reported in intact cAMP accumulation assays and much more closely approximates that seen in broken cell preparations (10).

TABLE 3

Agonist-induced alterations in isoproterenol competition curves for IPIN binding

% R_H, proportion of receptors binding agonist with a high affinity; K_L/K_H, ratio of the dissociation constants for the low (K_L) and high (K_H) affinity states of the receptor for agonists. % R_H and K_L/K_H were determined from computerized curve-fitting of the data to a two-site model (Ligand program) as described in the text. IC_{50} was determined as the mean ± geometric error of the mean from nonlinear regression of data points from each of 11 concentrations of isoproterenol (GraphPad Program ISI, Sigmoid Plot Subroutine). It should be noted that geometric means ± standard errors were calculated for all dissociation constants. The standard error values derived from nonlinear regression analysis shown here can only be viewed as estimates. The parameters were derived from pooled data from competition curves performed on five occasions.

	0.5 mM Mg^{2+}		10 mM Mg^{2+}	
	Control	Desensitized	Control	Desensitized
IC_{50} (nM)	45 (+6/−5)	196 (+19/−17)	17 (+4/−3)	160 (+13/−12)
%R_H	49 ± 6	31 ± 3	56 ± 5	32 ± 4
K_H (nM)	3.0 (+1.6/−1.1)	6.2 (+10.9/−4.0)	1.4 (+0.6/−0.4)	4.9 (+5.9/−2.7)
K_L (nM)	166 (+55/−41)	263 (+101/−73)	172 (+59/−44)	247 (+43/−33)
K_L/K_H	55	42	123	50
IC_{50} with Gpp(NH)p (nM)	200 (+20/−17)	281 (+25/−24)	244 (+26/−23)	280 (+24/−21)

lators (e.g., PGE_1), and/or to other more direct G_s stimulators (e.g., NaF, Gpp(NH)p); 2) the presence or absence of sequestration as a prominent, but secondary event; and 3) the role of direct stimulators of cAMP protein kinase in partially mimicking the effect of β-agonists. Receptor phosphorylation has been demonstrated to occur in both systems (reviewed in Ref. 30).

We found that desensitization of lymphocyte β-adrenergic adenylate cyclase activity follows a homologous pattern. Furthermore, in permeabilized lymphocytes desensitization may not be detected when studied at high Mg^{2+} levels. This is consistent in part with the recent finding of Clark *et al.* (6, 7). However, our findings differ in several important aspects. The studies of Clark in S_{49} lymphoma cell membrane fractions utilized 50 nM epinephrine and demonstrated a pattern characteristic of heterologous desensitization. At this concentration of epinephrine, functional desensitization of the lymphocyte β-receptor is small and inconsistent.[3] Further, Clark *et al.* (6) reported that, when assayed with high Mg^{2+} concentrations, the effect of epinephrine pretreatment in reducing receptor affinity for agonists was blunted (6). In contrast, we have demonstrated that, when assayed at high magnesium concentrations, agonist-induced alterations in competition curve parameters (IC_{50} for isoproterenol, K_L/K_H, %R_H) are still detectable at Mg^{2+} levels where no reductions in maximal isoproterenol-stimulated adenylate cyclase activity are detectable. Thus, there are substantial differences in the pattern of findings found by Clark *et al.* in their heterologous desensitization model in S_{49} lymphoma cell β-receptors and those we have presented in a homologous desensitization model in lymphocyte β-receptors. However, our data, together with those of Clark *et al.*, do suggest that agonist-induced alterations in adenylate cyclase sensitivity for Mg^{2+} may represent a mechanism common to both homologous and heterologous desensitization. In this regard, desensitization may be viewed as a process by which persistant receptor activation reduces the subsequent affinity of the β-receptor/adenylate cyclase complex for Mg^{2+}. Again, the concept that receptor activation modulates its own magnesium requirement is not unprecedented. Iyengar and Birnbaumer (4) previously reported that glucagon receptor activation results in a "leftward shift" in the apparent EC_{50} of Mg^{2+} for agonist-stimulated adenylate cyclase activation. Further, in several models, inhibitory receptor activation increases the EC_{50} of Mg^{2+} for adenylate cyclase activation (5). The present studies suggest an analogous effect with chronic β-agonist exposure.

Might changes in magnesium concentration modulate desensitization *in vivo*? To be of physiological importance, alterations in Mg^{2+} concentration should effect the expression of desensitization in terms of the final physiological outcome of β-adrenergic activation. Our data suggest that higher Mg^{2+} concentrations blunt the ability to detect β-adrenergic desensitization at the level of cAMP-dependent protein kinase. Therefore, the effect of Mg^{2+} on the expression of β-adrenergic desensitization is still apparent at least one step "distal" to adenylate cyclase activation. However, whether this effect is detectable at more distal points in the pathway of β-adrenergic signal transduction remains unproven.

It should also be recalled that β-adrenergic activation inhibits magnesium influx (31, 32) and, thus, modulates free intracellular Mg^{2+}. It has been suggested that this process could be important in the tonic regulation of adenylate cyclase activity. Our data would suggest that receptor-mediated alterations in Mg^{2+} flux might also be of potential importance in the regulation of desensitization. Overall, it should be stressed that the physiological significance of our findings (and those of Clark and Maguire and their colleagues) remains conjecture. However, these data certainly raise interesting hypotheses regarding the potential importance of alterations in free intracellular Mg^{2+} concentrations in modifying the expression of functional desensitization.

It must also be stressed that the molecular correlate for this change in Mg^{2+} EC_{50} has not been addressed. The effect described in these studies appears to be β-receptor specific and, thus, might be thought to reside in the receptor or receptor-G protein interaction. However, the data suggest that the site of this effect must be separate from the site at which Mg^{2+} regulates formation of the high affinity state of the receptor for agonists. Further, the alteration in β-adrenergic receptor sensitivity for Mg^{2+} would not appear to be related to any alterations in the process of agonist-induced receptor sequestration. Where the site of this defect may reside among the components of the β-receptor adenylate cyclase complex will be the focus of future studies.

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[3] R. Feldman, unpublished observation.

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