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Independent Desensitization of β-Adrenergic Receptor-Regulated Magnesium Transport and Cyclic AMP Accumulation

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SUMMARY

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We have previously shown that β-adrenergic inhibition of Mg^{2+} transport in cultured S49 lymphoma cells is not mediated by cyclic AMP (Maguire, M. E., and J. J. Erdos. *J. Biol. Chem.* **255:** 1030–1035 (1980). We now report that the rate of Mg^{2+} but not Ca^{2+} influx is decreased immediately upon the addition of (−)-isoproterenol and remains decreased at a constant rate for approximately 25 min in both wild-type and *kinase*$^-$ clones in the continued presence of agonist. The rate of Mg^{2+} influx gradually increases between 30 and 60 min, and complete desensitization to (−)-isoproterenol occurs after about 60–70 min. In contrast, cyclic AMP accumulation after the addition of (−)-isoproterenol peaks within 3 min in wild-type S49 cells. The calculated rate of cyclic AMP synthesis in intact wild-type cells falls to zero after 3 min of incubation even though biologically active agonist is still present at a high concentration. This acute desensitization is not due to activation of phosphodiesterase since identical time courses are obtained either in wild-type cells with a phosphodiesterase inhibitor or in *kinase*$^-$ S49 cells which have little or no detectable phosphodiesterase activity. These results indicate that β-adrenergic stimulation of cyclic AMP accumulation and therefore adenylate cyclase desensitizes independently of β-adrenergic inhibition of Mg^{2+} influx. Thus, not only is inhibition of Mg^{2+} transport by β-adrenergic receptor occupancy not mediated by cyclic AMP, but this receptor-modulated transport event appears to be regulated independently of adenylate cyclase.

INTRODUCTION

It is usually assumed that a given hormone receptor exerts its physiological action upon its cell through a single mechanism or pathway, that is, a hormone receptor population is coupled to a single effector system. In the case of catecholamines acting via a β-adrenergic receptor, the documented hormone effect is the activation of adenylate cyclase and a subsequent increase in intracellular cyclic AMP. We have recently described an effect of β-adrenergic receptor occupancy in S49 lymphoma cells not mediated by cyclic AMP, the inhibition of Mg^{2+} transport (1–3). Ca^{2+} uptake is not altered. This alteration in ion transport is not mediated by cyclic AMP as shown by experiments with variant clones of S49 cells[+] which lack specific components of the β-adrenergic receptor–adenylate cyclase–protein kinase system (2, 3). The mechanism of a β-adrenergic receptor-initiated response not mediated by cyclic AMP may be explained in two ways: The response observed could be a step in adenylate cyclase activation and thus prior to cyclic AMP generation, or alternatively, the response could be mediated by a completely different effector pathway independent of adenylate cyclase. We now present evidence that β-adrenergic activation of cyclic AMP synthesis and inhibition of Mg^{2+} transport desensitize with markedly different time courses. This indicates that these two effects of β-receptor occupancy are regulated independently and thus suggests that they may reflect independent mechanisms coupled to a single response population.

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MATERIALS AND METHODS

^{28}Mg (25–75 Ci/mol) was obtained from Brookhaven

[1] Abbreviations used: wild-type S49 cells, clone 24.3.2 of the S49 murine lymphoma; *kinase*$^-$ cells, clone 24.6.1 of S49 which phenotypically lacks the cyclic AMP-dependent protein kinase; phosphodiesterase, 3′:5′-cyclic AMP 5′-nucleotidase; R0 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone.

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National Laboratories. *Kinase*$^-$ cells[1] were obtained from Dr. Philip Coffino. Wild-type and *kinase*$^-$ S49 cells were grown and both cell isolation and ^{28}Mg uptake were performed as previously described (1, 4). Cyclic AMP assays were performed under conditions identical to those used for ^{28}Mg uptake except that the experiment was terminated by the addition of cold trichloroacetic acid to a final concentration of 5%. After centrifugation to remove precipitated protein, the cyclic AMP was purified by absorption on a Dowex-1-formate column (0.8 × 5 cm) which was washed with 5 ml of 0.1 N formic acid before cyclic AMP elution with 4 ml of 2 N formic acid. After lyophilization the samples were resuspended in 50 mM sodium acetate (pH 4.0) for assay of cyclic AMP by protein binding (5). Control experiments showed that the trichloroacetic acid was removed by the Dowex column and did not interfere with the assay. Recovery of cyclic AMP was measured by the addition of aliquots of ^3H-cyclic AMP to the acid cell extract. The values presented are total (intracellular plus medium) cyclic AMP.

The bars in each figure denote the SD of triplicate determinations. Where no bar is shown, the SD was smaller than the size of the symbol. Uptake of ^{28}Mg in each aliquot was individually corrected for isotopic decay (half-life, 21.3 h). The observed effect of (−)-isoproterenol or prostaglandin E_1 on ^{28}Mg uptake is due to decreased influx of Mg^{2+}, since the rate of efflux is unaltered by the presence of hormone and the half-time of efflux is greater than 70 min (unpublished data). The apparent rate of uptake is linear with respect to time for 20–25 min after the addition of hormone (1–3). Thus measurement of ^{28}Mg uptake over sequential 10-min (not shown) or 15-min intervals is a close approximation to the initial rate of ^{28}Mg influx.

RESULTS

In the absence of a phosphodiesterase inhibitor, wild-type S49 cells show a brief burst of cyclic AMP accumulation in response to either the β-adrenergic agonist (−)-isoproterenol or prostaglandin E_1 (Fig. 1). This accumulation appears to terminate rapidly, and cyclic AMP levels quickly fall even in the continued presence of agonist. If the apparent rate of cyclic AMP synthesis is determined by dividing the net cyclic AMP accumulated between successive time points by the time interval, the calculated rate of synthesis appears to fall to a basal rate within 2–3 min (Fig. 1, inset). This acute desensitization may be explained by three possibilities: (a) The agonist is being quickly destroyed, (b) the burst of cyclic AMP accumulation activates phosphodiesterase which then degrades the cyclic AMP as soon as it is synthesized, or (c) adenylate cyclase is being rapidly deactivated directly. In S49 cells, the first possibility does not occur since Shear *et al.* (6) have shown that (−)-isoproterenol is not extensively degraded even after 24 h of incubation. The second explanation, activation of phosphodiesterase, can be eliminated by two sets of experiments. Figure 2 shows that the maximal cyclic AMP accumulation in response to (−)-isoproterenol is increased about 2.5-fold in wild-type S49 cells if a phosphodiesterase inhibitor (0.1 mM R0 20-1724) is added. The K_a for (−)-isoproterenol is not significantly altered. If the experiment of Fig. 1 is repeated in the presence of R0 20-1724, the total cyclic AMP accumulation is greater in the presence of the inhibitor but accumulation still stops after 3–4 min (data not shown). Calculation of the apparent rate of cyclic AMP accumulation shows a time course quantitatively similar to that in the inset to Fig. 1. Thus the inhibition of phosphodiesterase activity does not alter the apparent desensitization of adenylate cyclase.

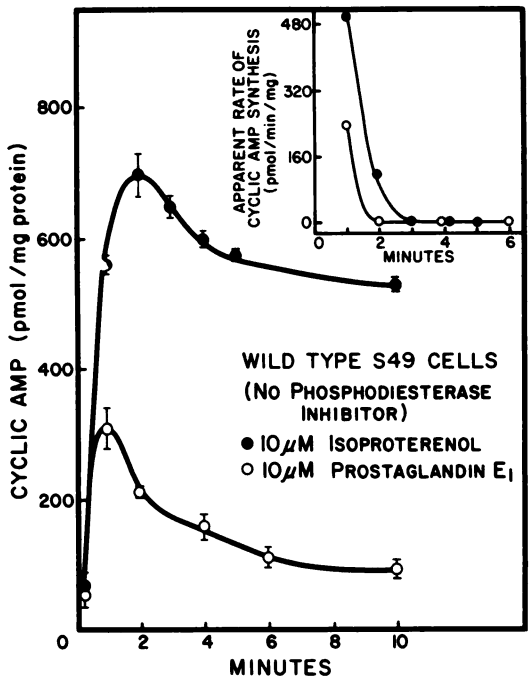

FIG. 1. *Time course of cyclic AMP accumulation and rate of synthesis (inset) in wild-type S49 cells in the absence of a phosphodiesterase inhibitor*

Wild-type S49 cells were washed twice by centrifugation in incubation buffer (1), resuspended at 5–8 × 10^6 cells/ml, and equilibrated for 10 min at 37°C in a shaking water bath before the addition of (−)-isoproterenol or prostaglandin E_1 to final concentrations of 10 μM each. Triplicate 1-ml aliquots were taken at various times and immediately added to tubes containing 0.1 ml trichloroacetic acid. Total cyclic AMP (cells plus medium) was determined as described in Materials and Methods. The apparent rate of cyclic AMP synthesis was calculated by determining the net difference in cyclic AMP accumulated between two successive time points and dividing by that time interval.

Experiments with a *kinase*$^-$ mutant of S49 offer further evidence for this interpretation. The *kinase*$^-$ clone used lacks the catalytic subunit of the cyclic AMP-dependent protein kinase (7, 8). The resulting inability of cyclic AMP to regulate cellular activity is manifest in alterations of numerous properties of this S49 clone. Most important for this study is the lack of cyclic nucleotide phosphodiesterase activity. Phosphodiesterase is an enzyme activity inducible by cyclic AMP in the S49 lymphoma. In S49 cells in which the total content of cyclic nucleotide-dependent protein kinase is decreased 80–90%, phosphodiesterase activity is reduced 60–80% (9, 10). The *kinase*$^-$ clone of S49 cells used in this study has no kinase catalytic unit at all (8) and would be expected to have virtually no phosphodiesterase activity. This is functionally confirmed by the data in Fig. 3. Unlike wild-type cells, the presence or absence of a phosphodiesterase

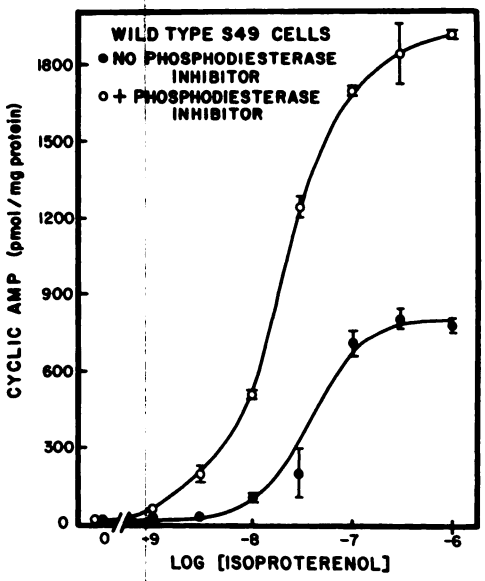

FIG. 2. *(−)-Isoproterenol dose-response curves for cyclic AMP accumulation in wild-type S49 cells*

Cells were washed as described in Fig. 1 and resuspended in incubation buffer with or without 0.1 mM R0 20-1724. Cells were incubated for 10–15 min at 37°C before triplicate 1.0-ml aliquots were added to tubes containing 10 μl of the appropriate (−)-isoproterenol concentration in 1 mM HCl. After 10 min of further incubation, the reaction was stopped with 0.1 ml trichloroacetic acid and the cyclic AMP determined as in Materials and Methods.

inhibitor has no effect on the maximal cyclic AMP accumulated in response to (−)-isoproterenol. Correspondingly, the time course of cyclic AMP accumulation (Fig. 4) peaks rapidly, but the total amount of cyclic AMP present does not decline even after 60 min, indicating the lack of significant phosphodiesterase activity. The calculated rate of cyclic AMP synthesis is quantitatively similar to that determined in wild-type cells (inset, Fig. 4). We conclude that β-adrenergic agonist and prostaglandin E_1 stimulation of adenylate cyclase in intact S49 cells rapidly desensitizes and that this acute desensitization is due to a decline in the rate of cyclic AMP synthesis rather than degradation of cyclic AMP or inactivation of hormone.

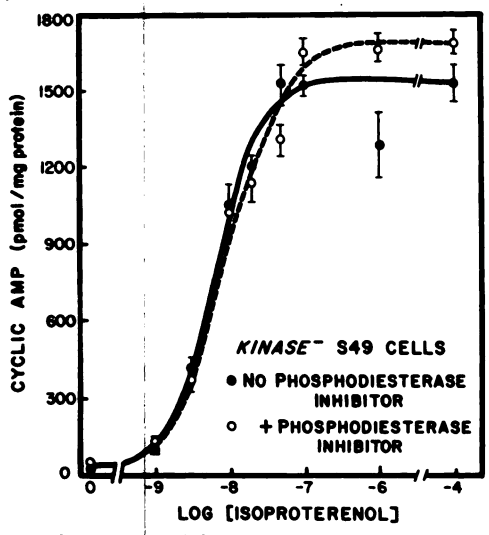

FIG. 3. *(−)-Isoproterenol dose-response curves for cyclic AMP accumulation in kinase⁻ cells*

See Fig. 2 and Materials and Methods for experimental details.

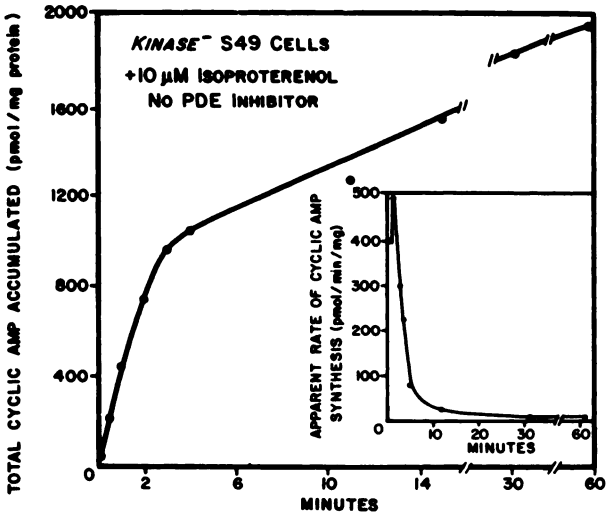

FIG. 4. *Time course of cyclic AMP accumulation and rate of synthesis (inset) in kinase⁻ cells*

See Fig. 1 and Materials and Methods for experimental details.

Since this acute desensitization is the result of inactivation at the level of the receptor–adenylate cyclase complex, it was of interest to determine if the β-adrenergic receptor-mediated inhibition of the Mg^{2+} influx also desensitized and, further, to compare its time course with that for desensitization of the cyclase response. The rate of influx of Mg^{2+} can be approximated (see Materials and Methods) by measuring ^{28}Mg uptake over sequential 15-min periods. Figure 5 shows an experiment in which aliquots of wild-type S49 cells from a single flask were added at various times to tubes containing ^{28}Mg and

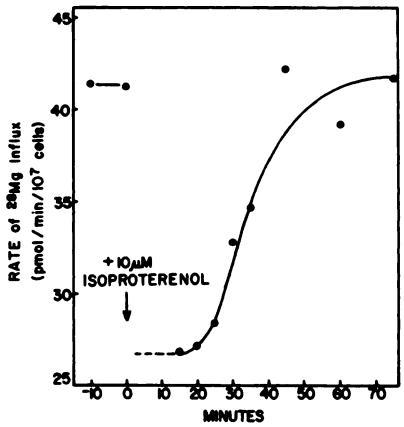

FIG. 5. *Time course of desensitization for (−)-isoproterenol inhibition of Mg^{2+} influx*

Wild-type S49 cells were washed twice by centrifugation and resuspended in incubation buffer containing 0.1 mM R0 20-1724 for 10 min at 37°C in a shaking water bath. At the time designated $t = 0$, the flask of cells was made 10 μM in (−)-isoproterenol by the addition of 0.001 vol of 10 mM drug. At the indicated times before and after (−)-isoproterenol, 4-ml aliquots were added to tubes containing 25 μl ^{28}Mg. After 15 min of further incubation, 1-ml aliquots were withdrawn from these tubes and the initial rate of ^{28}Mg influx was estimated as described in Materials and Methods.

influx was measured by determining cell-associated ^{28}Mg 15 min later. At the time indicated, the flask of cells was made 10 μM in (−)-isoproterenol. The data show that for at least 25 min after drug addition, the rate of ^{28}Mg influx is constant. After about 25 min the apparent rate of ^{28}Mg influx increases slowly so that by 60 min the effect of (−)-isoproterenol on ^{28}Mg influx has been completely abolished, and influx has returned to the rate observed before the addition of agonist. The same experiment in *kinase*$^-$ cells gives similar results, thus obviating any effect of phosphodiesterase activity on desensitization. In Fig. 6, the normalized data from such an experiment in *kinase*$^-$ cells are compared with the cyclic AMP data from Fig. 4. Cyclic AMP synthesis, and thus adenylate cyclase activity, clearly desensitizes much more rapidly than does Mg^{2+} influx. Thus, the desensitization of these two effects of β-adrenergic receptor occupation may be regulated independently.

The lack of cyclic AMP mediation of Mg^{2+} influx does not preclude the possibility that cyclic AMP causes the desensitization of ^{28}Mg influx observed in Fig. 5. The time courses shown in Fig. 6 are compatible with this speculation, although a protein kinase-mediated cyclic AMP effect is presumably ruled out by the demonstration of desensitization in *kinase*$^-$ cells which lack the cytosolic cyclic AMP-dependent protein kinase catalytic subunit. We would predict, if cyclic AMP causes desensitization of Mg^{2+} influx either directly or by a cytosolic or membrane-bound kinase activation, that pretreating wild-type S49 cells with cyclic AMP would prevent the (−)-isoproterenol inhibition of Mg^{2+} transport. We have previously shown that this is not the case (3) since incubation of S49 cells with 8-bromo cyclic AMP for 70–80 min leads to a greatly increased intracellular concentration of cyclic nucleotide but does not prevent a normal degree of inhibition of ^{28}Mg influx by (−)-isoproterenol. Since the 8-bromo analogue of cyclic AMP is a full agonist in S49 cells, desensitization of Mg^{2+} influx is not mediated by cyclic AMP, strengthening the conclusion that these two observed effects of β-receptor occupancy are regulated independently in S49 cells.

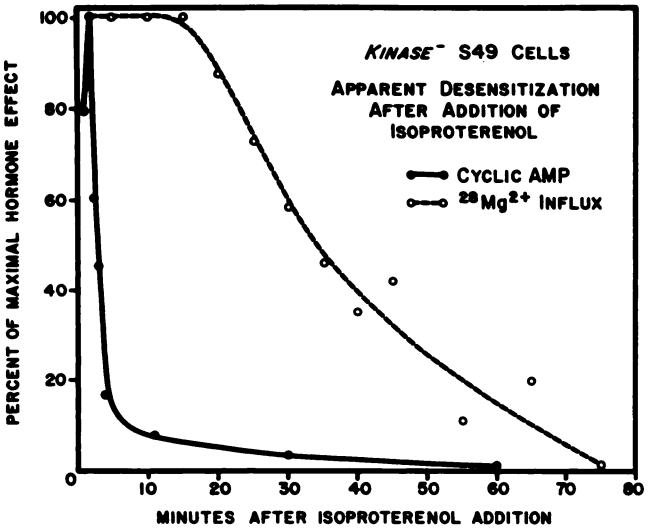

FIG. 6. *Comparison of time courses of desensitization of (−)-isoproterenol inhibition of Mg^{2+} influx and activation of adenylate cyclase in kinase*$^-$ *S49 cells*

The cyclic AMP data are redrawn from Fig. 4 normalized to the maximal calculated rate of cyclic AMP synthesis. The Mg^{2+} influx data are from an experiment identical to that shown in Fig. 5 except that *kinase*$^-$ cells were used in the absence of a phosphodiesterase inhibitor. The data are normalized to the maximal (−)-isoproterenol inhibition of the rate of ^{28}Mg influx.

DISCUSSION

We have previously shown that the effect of β-adrenergic agonists on Mg^{2+} transport is not mediated by cyclic AMP (2, 3). Further, the population of β-receptors involved in Mg^{2+} transport has the same pharmacological characteristics as that involved in activation of adenylate cyclase (3). This report demonstrates that the β-adrenergic receptor-mediated activation of adenylate cyclase and the β-adrenergic receptor-regulated inhibition of $^{28}Mg^{2+}$ influx densensitize with markedly different time courses. The question therefore arises as to the components involved in the mediation of these two responses. For example, these two effects could be the result of a single receptor population coupled to completely independent effector systems, or alternatively, both effects could be mediated by the known components of the receptor-adenylate cyclase system. Further, although the β-adrenergic receptors involved in both effects are pharmacologically identical, functionally independent subpopulations could exist, each mediating a single effect. Currently, there are believed to be three or possibly four known proteins that form the receptor–cyclase complex in the S49 lymphoma (11–14), a hormone receptor (R), one (11–13) or possibly two (14) nucleotide coupling factors (G/F), and a catalytic subunit (C). Our previous data (1–3) indicate that actions of R alone or G/F plus C alone are inadequate to explain β-adrenergic inhibition of Mg^{2+} transport. R alone is insufficient because receptor occupation in *unc* or *cyc*$^-$ S49 cells, in which R is uncoupled from G/F and C, does not inhibit ^{28}Mg accumulation. G/F plus C alone are insufficient since their activation by cholera toxin does not inhibit Mg^{2+} transport. The G/F moiety relevant to the latter would appear to be the membrane protein ADP-ribosylated by cholera toxin (14, 15) and associated with C. Recent data suggest that a second class of nucleotide coupling protein may exist (14, 16); its potential role, if any, with regard to Mg^{2+} transport is unknown.

Thus, our data on Mg^{2+} transport could indicate one of three possibilities for mediation of hormone-sensitive Mg^{2+} transport: (i) The entire receptor–cyclase complex is required and hormonal modulation of Mg^{2+} flux is possibly a mechanistic byproduct of activation of adenylate cyclase; (ii) R plus a nucleotide coupling protein associated specifically with R mediate the effect on Mg^{2+} uptake; or (iii) R is coupled to an as yet unidentified membrane component and this combination, independent of G/F(s) and C, modulates Mg^{2+} transport.

While our data are compatible with all three possibilities and more structural data are necessary, the latter two explanations seem more likely on the basis of the data in this report. If the entire receptor–cyclase complex is required for Mg^{2+} transport, it is difficult to envision a mechanism that would allow loss of catalytic activity but

not transport inhibition while retaining an intact complex. This seems more likely to occur either if R is associated with an independent G/F protein not connected with the G/F·C complex or if R is associated with an independent Mg^{2+} transport moiety. The existence of an independent transport protein(s) coupled to R may be the more likely of the latter two possibilities since Gilman and colleagues have demonstrated that G/F reconstituting activity is not tightly associated with the membrane. This reconstituting activity is able to reconstitute a membrane-associated hormone-sensitive adenylate cyclase without being itself stably incorporated into the membrane; hence, its involvement in any process requiring physical movement of mass through the membrane seems unlikely. Such speculation obviously requires further data. We are approaching this question by attempting to show Mg^{2+} transport and adenylate cyclase activity in sealed vesicles constructed with reconstituted components of the complex.

Our demonstration of two independent β-adrenergic receptor-mediated events in a homogeneous cell population which desensitize independently suggests the possibility that these two effector systems are differentially regulated. This may in turn suggest a potential explanation for some heretofore puzzling effects of some interactions between different hormones. In several cell systems it has long been known that insulin or α-adrenergic catecholamines might either oppose (17–19) or mimic β-adrenergic activation (20, 21), depending on the cell system examined. The mechanism of interaction between such hormones with opposing effects has not been satisfactorily explained by opposing effects on cyclic AMP concentration. We propose that Mg^{2+} transport may be involved in this interaction. It has been reported that insulin (22, 23) and α-adrenergic agonists (24, 25) stimulate Mg^{2+} accumulation in uterine smooth muscle and fat cells. Our demonstration that β-adrenergic agonists inhibit Mg^{2+} influx in S49 cells and that this appears to be an independently regulated pathway of hormone response suggests that at least some hormones which interact with β-adrenergic receptor-mediated events may do so not through cyclic AMP but through regulation of Mg^{2+} transport.

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