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Regulation of Cyclic AMP Accumulation in Lymphoid Cells[1] (42125)

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Abstract. We have examined several features of the regulation of cyclic AMP accumulation in lymphoid cells isolated from peripheral blood of human subjects and in the murine T-lymphoma cell line, S49. S49 cells are unique because of the availability of variant clones with lesions in the pathway of cyclic AMP generation and response. We found that human lymphoid cells prepared at 4°C showed substantially greater cyclic AMP accumulation in response to histamine and the β-adrenergic agonist isoproterenol than did cells prepared at ambient temperature. The muscarinic cholinergic agonist carbamylcholine and peptide hormone somatostatin failed to inhibit cyclic AMP accumulation in human lymphoid cells and treatment with pertussis toxin (which blocks function of G_i, the guanine nucleotide binding protein that mediates inhibition of adenylate cyclase) only minimally increased cyclic AMP levels in these cells. Thus the G_i component of adenylate cyclase appears to play only a small role in modulating cyclic AMP levels in this mixed population of lymphoid cells. Incubation of whole blood with isoproterenol desensitized human lymphocytes to subsequent stimulation with β agonist. This desensitization was associated with a redistribution of β-adrenergic receptors such that a substantial portion of the receptors in intact cells could no longer bind a hydrophilic antagonist. Wild-type S49 lymphoma cells showed a similar redistribution of β-adrenergic receptors after a few minutes' incubation with agonist. Based on studies in S49 variants, this redistribution is independent of components distal to receptors in the adenylate cyclase/cyclic AMP pathway. By contrast, a more slowly developing, agonist-mediated down-regulation of β-adrenergic receptors was blunted in variants with defective interaction between receptors and G_s, the guanine nucleotide binding protein that mediates stimulation of adenylate cyclase. Unlike results in human lymphoid cells, S49 cells show a prominent inhibition of cyclic AMP accumulation mediated by G_i; this inhibition is promoted by somatostatin and blocked by pertussis toxin. Inhibition by G_i is unable to account for the marked decrease in ability of the diterpene forskolin to maximally stimulate adenylate cyclase in S49 variants having defective G_s. These results emphasize that both G_s and G_i component are important in modulating cyclic AMP accumulation and receptors linked to adenylate cyclase in S49 lymphoma cells. Although human peripheral blood lymphocytes demonstrate only a small involvement of G_i under basal condition, the prominent role of this component in S49 cells suggests that certain subpopulations of lymphoid cells might be particularly enriched in activity of this component.
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Ever since Sutherland and co-workers discovered that adenosine 3'5'monophosphate (cyclic AMP, cAMP) was a second messenger for membrane-active hormones (1), a large number of investigators have undertaken studies to define the repertoire of extracellular signals that alter cyclic AMP levels in particular cell types. For lymphoid cells, a detailed understanding of either the identity or mechanisms of action of such signals is not yet available. Cyclic AMP has been implicated in the regulation of a variety of immune and inflammatory responses (2, 3), but the definitive role of this nucleotide in regulating lymphoid cell function has been difficult to discern. There are probably several reasons for this difficulty, among them: (a) The many subpopulations of lymphoid cells are likely to differ in their expression of receptors linked to stimulation and inhibition of adenylate cyclase and in their endogenous substrates for cyclic AMP-mediated protein phosphorylation; (b) cells at different stages in lymphoid cell differentiation show qualitative and quantitative differences in hormonal and cyclic AMP responses; (c) interspecies differences in responses have made it difficult to generalize regarding these responses; and (d)

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our increased understanding of the receptor-linked cyclic AMP pathway indicates that it is a complex, multicomponent system, subject to regulation at many loci. As reviewed elsewhere (4, 5), recent work indicates that stimulation and inhibition of adenylate cyclase are mediated via two homologous guanine nucleotide binding proteins, termed G_s and G_i, respectively.

Our laboratory has taken two approaches to examining the regulation of cyclic AMP accumulation in intact lymphoid cells. In the first, we have recently begun studies to explore the regulation of cyclic AMP generation of human peripheral blood mononuclear cells (predominantly lymphocytes) by β-adrenergic agonists and other compounds. In the second, we have utilized a cultured cell line, the murine T-lymphoma S49, to help define mechanisms regulating cyclic AMP synthesis (6, 7). S49 cells have proved uniquely useful for such studies because a variety of S49 variants have been isolated that have defects in the pathway of cyclic AMP generation and response. In this report, we present some of our recent findings in each of those two systems related to stimulation and inhibition of cyclic AMP generation.

Methods. Human peripheral blood mononuclear cells were isolated from healthy, ambulatory subjects (ages 18–40) using the technique of Boyüm (8) with minor modifications. The principal modification was to conduct the centrifugation and isolation of cells at 4°C, whereas most investigators perform these procedures at ambient temperature. As indicated in the results, isolation of mononuclear cells at 4°C leads to cells with enhanced cyclic AMP accumulation. All cells were used immediately following isolation because prolonged storage of the cells at either 4°C or ambient temperatures lowered cyclic AMP accumulation. Wright's stain of the preparation indicates that >80% of the cells are lymphocytes; the rest are monocytes.

S49 lymphoma cells were grown in suspension in Dulbecco's modified Eagle's medium plus 10% horse serum (heat inactivated), routinely in 250 ml T-flasks to a density of $1–2 \times 10^6$ cells/ml. Viability was assessed by analysis on a Coulter cell counter and channelyzer or by trypan blue exclusion. Prior to use in experiments, cells were routinely centrifuged at 300*g* for 5 min and resuspended in fresh medium.

In studies of cyclic AMP generation, cells were incubated with the cyclic nucleotide phosphodiesterase inhibitor Ro20-1724 (0.1 m*M*); with human lymphocytes, 0.1 m*M* isobutylmethylxanthine (another phosphodiesterase inhibitor) was included as well. Cyclic AMP levels were assayed in cell pellets obtained by spinning cells and media (in a Beckman microfuge for 30 sec), aspiration of media, addition of 50 m*M* Na-acetate (pH 4.0)/0.2 m*M* isobutylmethylxanthine, and boiling samples for 3–5 min. We assayed cyclic AMP in aliquots of the boiled samples using a competitive binding protein method (9). Studies conducted with isoproterenol included 10–20 μg/ml catalase and superoxide dismutase in order to block oxidation of the agonist (9). Forskolin was dissolved in 95% ethanol and added to cells so that final ethanol concentration was <0.1%. Data are expressed as picomole cyclic AMP generated/10^7 cells and unless indicated otherwise represent results typical of those obtained in at least two replicate experiments.

β-Adrenergic receptors were assayed in intact human lymphocytes or S49 lymphoma cells using [^{125}I]iodocyanopindolol in radioligand binding assays conducted as previously described (9), except that studies with human lymphocytes were conducted at 4°C for 18 hr. In assays of total cellular receptors, 1 μ*M* (−)-propranolol (a hydrophobic β-adrenergic antagonist) was used to define nonspecific binding. In studies assessing redistribution of β-receptors in human lymphocytes, CGP-12177 (a hydrophilic β-adrenergic antagonist) was used to compete for [^{125}I]iodocyanopindolol sites. For S49 cells, [^{3}H]CGP-12177 was used to assess redistribution of β-receptors in studies conducted at 4°C for 18 hr (10).

Results. *Human peripheral blood lymphoid cells.* As indicated under Methods, we prepared peripheral blood lymphocytes at 4°C rather than at ambient temperature. Figure 1 shows the differences in results that were obtained in cells isolated in parallel at the two temperatures; cellular cyclic AMP accumulation was assessed after 2 min incubation at 37°C with 10 μ*M* of the β-agonist (−)-isoproterenol. Although we found considerable intersubject variability, we consistently

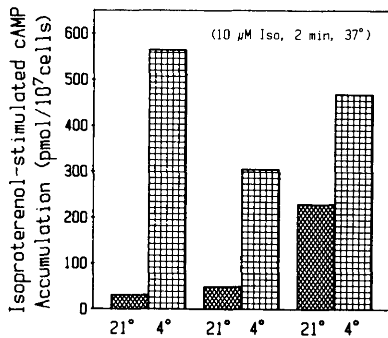

FIG. 1. Isoproterenol-stimulated cyclic AMP levels in human lymphocytes isolated at 4°C or at ambient temperature (21°C). Lymphocytes were isolated and washed from whole blood by the method of Boyüm (8) using centrifugations conducted at either 4° or 21°C. Data are shown for isoproterenol-stimulated cyclic AMP levels of lymphocytes from three different subjects. Incubations were begun by adding 0.1 ml of cells to 0.9 ml of buffer prewarmed to 37°C and containing isoproterenol (10 *μM*) and inhibitors of phosphodiesterase. Basal cyclic AMP levels were subtracted to obtain the results shown.

observed a much larger response to isoproterenol in cells prepared at 4°C. We obtained similar results when histamine was used instead of isoproterenol to raise intracellular cyclic AMP levels. When cells were prepared at 21°C and then cooled for 1-3 hr, they did not recover the enhancement in cyclic AMP accumulation, whereas cells prepared at 4°C and then warmed had a loss in cyclic AMP accumulation. The amount of isoproterenol-stimulated cyclic AMP accumulation on a per-cell basis in human peripheral blood cells prepared at 4°C (Fig. 1) approximates that of S49 cells (typically 300–1000 pmol/10^7 cells).

Several neurotransmitters and hormones inhibit cyclic AMP accumulation via receptors linked to G_i, the inhibitory guanine nucleotide binding protein that regulates adenylate cyclase activity (11). The function of G_i is blocked by a toxin from *Bordatella pertussis,* pertussis toxin, which was originally termed "islet activating protein" (12). Lymphoid cells isolated from blood that had been incubated with 100 ng/ml pertussis toxin for 4 hr showed a two- to fourfold enhancement in basal levels of cyclic AMP and only a small increase in response to isoproterenol. From these results, it would appear that this mixed population of human lymphocytes has a low tonic activity of the G_i unit of adenylate cyclase. In addition, we asked whether the peptide hormone somatostatin or the cholinergic agonist carbamylcholine, which acts via G_i in other cell types (12–14), would inhibit cyclic AMP accumulation in human lymphocytes. Neither somatostatin (0.1 *μM*) nor carbamylcholine (10 *μM*) altered cyclic AMP levels in human lymphocytes.

An important determinant of cellular response to hormones and neurotransmitter in a variety of cell types is desensitization (also termed tolerance, tachyphylaxis, refractoriness, or deactivation). Previous work has indicated that human subjects treated with β agonists show both a desensitization of cyclic AMP accumulation and a down regulation of β receptors [e.g., (15)]. In our approach to examining desensitization of human lymphoid cells, we incubated freshly isolated whole blood at 37°C with the β-agonist isoproterenol and then assessed expression of β-adrenergic receptors in both radioligand binding and cyclic AMP accumulation assays. As shown in Fig. 2, cells isolated from blood incubated in this manner have a prominent decrease in their ability to

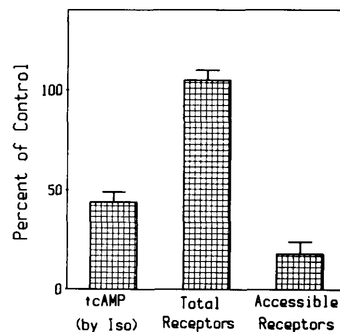

FIG. 2. β-Adrenergic desensitization of normal human lymphocytes. Whole blood was incubated with or without 1 *μM* isoproterenol at 37°C for 10 min. The blood was then cooled to 4°C, lymphocytes were isolated, and cells were used to assess isoproterenol (10 *μM*) -stimulated cyclic AMP accumulation (left), total β-adrenergic receptors (specific binding of [^{125}I]iodocyanopindolol as determined using 1 *μM* (−)-propranolol to define nonspecific binding, (middle), or accessible receptors (redistribution of β-adrenergic receptors, [^{125}I]iodocyanopindolol binding sites that could be competed by 1 *μM* of the hydrophilic antagonist CGP-12177, right). All results are shown for desensitized cells as a percentage of results obtained from simultaneous controls.

accumulate cyclic AMP but retain a normal number of β-adrenergic receptors. However, these receptors appear to have become redistributed in the cells, and ~80% of β receptors in lymphocytes isolated from this blood are unable to bind CGP-12177, a hydrophilic β-adrenergic antagonist (Fig. 2).

S49 lymphoma cells. Experiments have been conducted in S49 cells to investigate mechanistic aspects of some of the results obtained in the human cells. There are some obvious differences in the two systems (murine versus human, homogeneous cell line versus heterogeneous cell types, "transformed" versus benign, etc.), but S49 cells have the advantage of providing a uniform, easily manipulable system in which variant cells can be used as "reagents" to assess the role of components known to be defective or absent in these variants. We conducted studies examining two issues: β-adrenergic receptor desensitization and the role of G_i in cyclic AMP accumulation.

As we found with human lymphoid cells, incubation of S49 cells with isoproterenol produced a rapid desensitization of cyclic AMP accumulation (Fig. 3), without a major decrease in β-adrenergic receptor number (9, 16–18). As was also noted with human lymphoid cells, S49 cells incubated with isoproterenol had a redistribution of receptors (Fig. 4, left panel) such that within 15 min only about 35% of receptors were still detectable in binding studies conducted with the hydrophobic antagonist [^{3}H]CGP-12177 (10, 18).

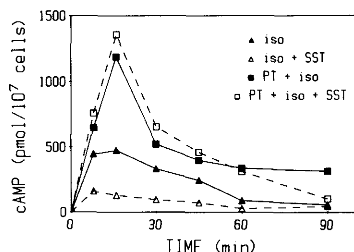

FIG. 3. Kinetics of isoproterenol-mediated cyclic AMP accumulation in wild-type S49 lymphoma cells: influence of somatostatin and pertussis toxin. Cells were incubated with or without 100 ng/ml pertussis toxin for 4 hr and then 0.1 mM Ro20-1724 and 1 μM (−)-isoproterenol were added alone or together with 0.1 μM somatostatin. Aliquots were assayed for cellular cyclic AMP levels at the indicated times.

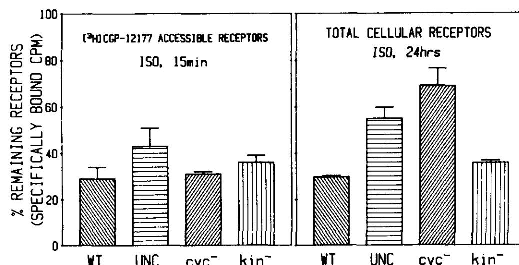

FIG. 4. Isoproterenol-induced redistribution and down regulation of β-adrenergic receptors in wild-type and variant S49 cells. In the left panel cells were incubated with or without 1 μM (−)-isoproterenol for 15 min, washed, and then incubated at 4°C for 18 hr with [^{3}H]CGP-12177 in the absence and presence of 1 μM (−)-propranolol to define nonspecific binding. In the experiments in the right panel, cells were incubated with 1 μM (−)-isoproterenol for 16–18 hr, washed three times, and then incubated with [^{125}I]iodocyanopindolol in the absence and presence of 1 μM (−)-propranolol. Data are presented as percentage of specific binding sites remaining relative to control cells for wild-type, cyc$^-$, UNC, and kin$^-$ S49 cells. Cyc$^-$ and UNC cells have defective interaction of β receptors with G_s, and Kin$^-$ cells lack cyclic AMP-dependent protein kinase activity.

Similar results were obtained with S49 variants having alterations in G_s or absent cyclic AMP-dependent protein kinase activity [Fig. 4, left panel, and Refs. (10, 18)]. Thus G_s and cyclic AMP-mediated protein phosphorylation are not required for receptor redistribution (10, 18).

When S49 cells are incubated with β agonists for many hours the number of β receptors decreases or down-regulates. In contrast to receptor redistribution, this down regulation of receptors is blunted in S49 variants having absent G_s or lesions in G_s which inhibit the ability of G_s to interact with β receptors [Fig. 4, right panel, and Refs. (16, 18)]. S49 cells that lack cyclic AMP-dependent protein kinase down-regulated β receptors as well as did wild-type cells. These results indicate that receptor redistribution and receptor down regulation in S49 cells (and by analogy, other lymphoid cells) are distinct processes that likely result from separate molecular interactions.

Although β receptors are known to mediate their effect via interaction with G_s, some recent data have indicated that purified receptors can interact with G_i when reconsti-

tuted in phospholipid vesicles (19). Is such an interaction physiologically relevant? We have undertaken studies to quantitate the potential importance of G_i activity in S49 cells; G_i in S49 membranes was recently detected by several other laboratories (1, 12, 20). As shown in Fig. 3, wild-type S49 cells show a prominent enhancement in isoproterenol-mediated cyclic AMP accumulation after cells were preincubated with 100 ng/ml pertussis toxin for 4 hr. However, the kinetics of isoproterenol-mediated cyclic AMP accumulation in cells incubated with pertussis toxin was quite similar to that of cells not incubated with the toxin. This suggests that although G_i may act as a tonic inhibitor of adenylate cyclase activity in S49 cells, linkage of β receptors to G_i seems unlikely to mediate desensitization in these cells. Moreover, as shown in Fig. 3, somatostatin is a prominent inhibitor of cyclic AMP accumulation in the S49 cells (unlike human lymphocytes) and pertussis toxin blocks this inhibition.

Some have proposed that G_i may also play a major modulatory role on other agents that increase cyclic AMP levels in the S49 system (21). For example, the diterpene forskolin substantially stimulates cyclic AMP accumulation in intact S49 cells, but this stimulation is markedly blunted in cells like the cyc$^-$ S49 variant, which lacks G_s (22). To test if the presence of an active G_i unit in cyc$^-$ cells could explain their failure to respond to forskolin, we incubated cyc$^-$ cells with pertussis toxin and then examined cyclic AMP accumulation in response to forskolin. As shown in Fig. 5, both wild-type and cyc$^-$ S49 cells treated with pertussis toxin had enhanced response to forskolin, but cyc$^-$ cells still accumulated far less cyclic AMP accumulation than did wild-type cells. This result implies that G_i activity in cyc$^-$ cells cannot explain the markedly lower response of these cells to forskolin; the absence of G_s in these cells provides a better explanation for their poor response to forskolin.

Discussion. We have characterized several factors that influence cyclic AMP levels in lymphoid cells: temperature of cell isolation, receptors linked to stimulation and inhibition of adenylate cyclase, and receptor desensitization. The combined use of cells from humans and from the S49 line provides com-

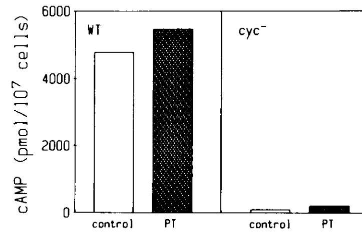

FIG. 5. Forskolin-stimulated cyclic AMP accumulation in wild-type and cyc$^-$ S49 lymphoma cells. Cells were incubated with or without 100 ng/ml pertussis toxin for 4 hr and then 50 μM forskolin and 0.1 mM Ro-20-1724 were added. Cellular cyclic AMP levels were assayed after 15 min incubation with forskolin.

plementary information regarding regulation of cyclic AMP accumulation in intact cells.

The mechanism by which lowering the temperature for isolation of lymphocytes from peripheral blood leads to enhanced cyclic AMP accumulation is unknown. One possibility is that disruption of the cytoskeleton, in particular disassembly of microtubules, leads to enhanced cyclic AMP accumulation. Lower temperatures are known to produce this disassembly and pharmacologic inhibition of microtubular assembly also enhances cyclic AMP accumulation in both normal lymphoid cells and S49 lymphoma cells (23, 24). However, because cells prepared at room temperature and then cooled to 4°C fail to show enhanced response, this suggests that factors other than, or in addition to, microtubular disassembly produce this enhancement.

Both human lymphoid cells and S49 cells possess β-adrenergic receptors that are linked to stimulation of adenylate cyclase, but whereas S49 cells demonstrate an inhibition of cyclic AMP accumulation in response to the hormone somatostatin, human lymphoid cells fail to show this. Perhaps human lymphoid cells lack somatostatin receptors but alternatively the poor response of human lymphocytes to pertussis toxin suggests that G_i may have only a limited role in modulating cyclic AMP in human lymphoid cells. Possible explanations would include human lymphoid cells have a low expression of G_i, the conditions used to assess response to pertussis toxin were inappropriate, or only a limited subset of lymphoid cells actively ex-

press G_i. Although we favor the latter explanation (since G_i has a prominent role in S49 cells), these and other explanations (e.g., species differences) will require further testing.

The results related to desensitization are of both physiologic and pharmacologic interest. Most previous data on desensitization to β catecholamines has involved studies of cultured cell lines or erythrocytes from birds and frogs [reviewed in (25, 26)]. The documentation that normal human cells also show a rapid desensitization to β catecholamine has been observed previously [e.g., (15)] but the molecular basis for this desensitization has not been defined. Our results documenting a redistribution of β-adrenergic receptors in human cells agrees with results obtained previously in several model systems (including S49 cells) (10, 25, 26). Redistribution of β receptors may represent an agonist-promoted internalization of receptors but this notion requires additional evidence. In preliminary studies we find that addition of exogenous epinephrine (instead of isoproterenol) to whole blood can also promote desensitization of cyclic AMP accumulation and a redistribution of β receptors in human lymphocytes and that concentrations of epinephrine effective in producing these effects are approaching those achieved in settings of excessive circulating catecholamines. Thus desensitization and receptor redistribution may be physiologically important mechanisms by which human lymphocytes modulate responsiveness to catecholamines, to which these cells are continuously exposed.

The studies in S49 cells indicate that redistribution of β-receptors does not require involvement of components in the adenylate cyclase/cyclic AMP pathway that are distal to β-adrenergic receptors, whereas the slower down-regulation of β receptors does require an intact G_s component (10, 16, 18). Thus the early and late phases of desensitization must involve discrete mechanisms. Key events involved in the early phase of desensitization in S49 cells remain to be defined but decreases in apparent affinity of β receptor for agonist are likely to be another feature of this process (9, 27). We are currently conducting further studies to define mechanisms mediating both phases of β-adrenergic receptor desensitization in S49 lymphoma and normal human lymphoid cells.

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