Human erythroleukemia cells are a model system for studies of α2-adrenergic receptors and their coupling to inhibition of adenylate cyclase (McKernan, R. M., Howard, M. J., Motulsky, H. J., and Insel, P. A. (1987) Mol. Pharmacol. 32, 258–265). Using Fura-2, we show that α2-adrenergic receptor stimulation also increases intracellular Ca2+ in these cells by 80–200 nm. Although epinephrine only inhibited forskolin-stimulated cAMP generation when β-adrenergic receptors were blocked, the Ca2+ increase was not affected by β-adrenergic receptor blockade. The Ca2+ increase was not affected by forskolin or β-bromo-cAMP. Thus, α2-adrenergic receptors independently couple to elevation of intracellular Ca2+ and adenylate cyclase inhibition. Chelating all extracellular Ca2+ did not reduce the response, demonstrating mobilization of intracellular, rather than influx of extracellular Ca2+. The epinephrine-stimulated Ca2+ mobilization occurred prior to any detectable increase in inositol-(1,4,5)-trisphosphate. It was abolished by pretreatment with pertussis toxin (which blocks some G protein-mediated processes), but not by aspirin and indomethacin (which inhibit cyclooxygenase), nordihydroguaiaretic acid (which inhibits lipooxygenase), or Na+/H+ free buffer (to block any Na+/H+ exchange). We conclude, therefore, that α2-adrenergic receptors on human erythroleukemia cells couple to mobilization of intracellular Ca2+ via a (pertussis toxin-sensitive) G protein-mediated mechanism that is independent of inhibition of adenylate cyclase.

α2-Adrenergic receptors couple to an inhibition of adenylate cyclase in every tissue studied. Many α2-adrenergic receptor-initiated responses, however, are not mediated by lowered levels of cAMP (1). Data presented in the last few years demonstrate that α2-adrenergic receptors are quite versatile and can also couple to other second messenger systems. Some data suggest that α2-adrenergic receptors in some cell types may activate Na+/H+ exchange (2, 3), stimulate flux through K+ channels (4, 5), or inhibit voltage-sensitive Ca2+ channels (6–8).

Human erythroleukemia (HEL)1 cells are a model system for studying α2-adrenergic receptors and their coupling to inhibition of adenylate cyclase (9). They were derived from the blood of a patient with Hodgkin’s disease and erythroleukemia and contain markers for platelets, B cells, and erythrocytes (10, 11).

Here we demonstrate that α2-adrenergic receptors on HEL cells couple to mobilization of intracellular Ca2+ by a pertussis toxin-sensitive mechanism that appears to be independent of cAMP formation, Na+/H+ exchange, Ca2+ influx, and IP3 production.

MATERIALS AND METHODS

Cell Culture

HEL cells were grown in RPMI 1640 medium supplemented with 2 mM glutamine and 10% newborn calf serum. Cells were maintained at 37 °C with 95% air, 5% CO2 at a density between 2 and 6 × 10⁶ cells/ml by daily dilution with fresh medium.

It should be noted that the cells used here differ in two ways from those used in the experiments published previously (9). The α2-adrenergic receptors are now virtually all located on the cell surface even after incubation with epinephrine, and the cells now express β-adrenergic receptors. However epinephrine also mobilizes Ca2+ in HEL cells matching the previous phenotype.

Ca2+ Measurements

Intracellular Ca2+ was assessed by measuring Fura-2 fluorescence using methods that have been described in detail elsewhere (12). Briefly, cells were loaded with Fura-2 acetoxyethyl ester (1 μM) for 1 h at room temperature in buffer containing 120 mM NaCl, 20 mM Hepes, 5 mM KH2PO4, 1 mM magnesium acetate, 1 mM CaCl2, and 1 mg/ml glucose at pH 7.4. The same buffer was used for experiments measuring cAMP, cGMP, and IP3. Cells were then washed twice and resuspended in fresh buffer. Fluorescence was measured at 25 °C with excitation at 340 nm and emission at 510 nm. Calibration to intracellular Ca2+ included corrections for the contribution of extracellular Fura-2.

Measurement of cAMP

10⁶ cells were incubated in a total volume of 1 ml buffer containing 0.1 mM isobutylmethylxanthine and 0.1 mM Ro 20-1724 for 2 min at 37 °C. Details of the procedure have been published (9).

Measurement of cGMP

5 × 10⁶ cells were incubated in a total volume of 1 ml buffer containing 0.5 mM isobutylmethylxanthine for 5 min at 37 °C in the presence or absence of epinephrine. Incubation was stopped by cen-

1The abbreviations used are: HEL, human erythroleukemia; IP3, inositol-(1,4,5)-trisphosphate; HPLC, high performance liquid chromatography; EGT, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

*This work was supported by National Institutes of Health Grants HL32632 and HL40387 and the American Heart Association with funds provided in part by the California affiliate. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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trifurging the cells, aspirating the supernatant, adding 150 μl of 2 mM isobutylmethylxanthine, 5 mM EDTA, 5 mM Tris, pH 7.5, and placing samples in a boiling water bath for 5 min. Tubes were centrifuged, and cGMP was assessed in aliquots of the supernatant using a commercially available radioimmunoassay.

Phosphatidylcholine Metabolites

Phosphatidylcholine metabolites were measured in three ways: HPLC analysis, by column elution, and by a competitive protein binding assay.

HPLC—HEPES cells were suspended at 8 x 10^6 cells/ml in isositol-free RPMI medium containing 10% fetal calf serum (previously dialyzed against phosphate-buffered saline) and 10 μCi/ml myo-[3H]-inositol. After a 22-h incubation period, the cells were washed once, resuspended in serum-free RPMI, and allowed to re-equilibrate for 1 h at 37°C before use. In the assay for inositol phosphate formation, 0.6-ml aliquots of radiolabeled HEL cells (6 x 10^6 cells) were incubated with either epinephrine or thrombin. The reaction was stopped by the addition of 0.3 ml of 12% perchloric acid containing 3 mM EDTA, 1 mM diethylstilbestrol, and 15 μg of bovine serum albumin. The precipitate was removed by centrifugation and re-extracted with perchloric acid. After recombining the supernatants, Hepes was added to a final concentration of 50 mM, and the pH was adjusted to 7.5 with KOH. After incubation on ice for at least 1 h, the precipitate which formed upon addition of the KOH was removed by centrifugation, and the sample volume was reduced to <1 ml using a Speed Vac Concentrator (Savant Instruments). Chromatography was performed on a Whatman Partisil-SAX anion exchange column using a linear 40-min gradient from 0 to 1.5 M ammonium formate, pH 3.7, at a flow rate of 1.5 ml/min. Samples were collected at 1-min intervals. Peaks corresponding to inositol 1-phosphate, inositol 2-phosphate, and the 1,4,5- and 1,3,4-isomers of IP_3 were identified using radiolabeled standards and quantitated by scintillation counting.

Column Elution—Cells were incubated 15-24 h in medium 199 (which contains only 0.3 μM inositol) supplemented with 5% fetal calf serum (previously dialyzed against phosphate-buffered saline) and 10 μCi/ml myo-[3H]-inositol (from American Radiochemicals, St. Louis, MO). Tritium incorporation was approximately 2 x 10^6 cpm/10^6 cells. The cells were then washed into buffer that included 10 mM LiCl. 10^6 cells were incubated in a final volume of 1 ml in the absence or presence of agonists at 25°C. The reaction was stopped after 5 min, and the cells were lysed by addition of 1 ml of methanol followed by 2 ml of chloroform. The tubes were vigorously vortexed. Aliquots (1.5 ml) of the upper phase were placed on columns containing 200 mg of Dowex AG 1-X8 (mesh 100-200). Inositol phosphates were eluted with 2 M ammonium formate, 150 mM formic acid according to standard methods (15).

Protein Binding—The mass of hormone-stimulated inositol-(1,4,5)-trisphosphate generation was determined with a competitive protein binding assay from Amersham Corp. After washing into buffer, ~500,000 cells were preincubated for 5 min at 37°C. Then 100 μl of agonist (prepared in buffer) was added to 100 μl of cells. After 10 s, the incubation was stopped by adding 20 μl of 30% perchloric acid and placing the tubes on ice. The mixture was neutralized by adding 35 μl of 36% HClO. The accumulated IP_3 was assayed at 4°C. The samples were incubated with binding protein and [3H]IP_3 for 15 min. We centrifuged the tubes, discarded the supernatants, resuspended the pellet in 200 μl of buffer which was transferred to scintillation vials containing 4.5 ml of scintillation fluid. Results were calibrated with a standard curve using 0.1-50 pmol of IP_3. The binding protein used cross-reacts with other inositol phosphates as follows (compared with IP_3 binding): 6.4% for inositol-tetrakisphosphate, 0.2% for inositol-(1,3,4,5)-tetraphosphate, and less than 0.1% with other inositol phosphates (data from Amersham Corp.).

Determination of GTP-binding Proteins

Western blots were performed using previously published techniques (14).

Materials

Fura-2 was purchased from Molecular Probes (Eugene, OR), myo-[3H]-inositol (specific activity 15 Ci/mmol) from American Radiochemicals (St. Louis, MO), [3H]GMP radioimmunoassay kit from Amersham Corp., forskolin from Calbiochem, pertussis toxin from List (Campbell, CA), nordihydroguaiaretic acid, oxymetazoline, yohimbine, and (-)-epinephrine from Sigma, and isositol-free RPMI medium from GIBCO. The following drugs were gifts of the respective companies: UK 14,304 (5-bromo-N-(4,5-dihydroimidazol-2-yl)-6-quinolinamine) from Pfizer (New York, NY), (-)-propranolol from Ayerst (New York), moxonidine and BE 2254 (2-[β-(4-hydroxyphenyl)butoxy]methyl)tetralone (1) from Biereich (Hamburg, West Germany), ARC 239 from Tomase (Biberach, West Germany), clonidine from Boehringer (Ingelheim, West Germany).

Data Presentation

Data are presented as mean and standard error of the mean. K_i values were calculated from the I_C50 values according to the equation given by Cheng and Prusoff (15).

RESULTS

Epinephrine transiently increased intracellular Fura-2 fluorescence as shown in Fig. 1. The fluorescence peaked between 20 and 40 s and returned completely to base line within 120 s. Basal concentrations of intracellular Ca^{2+} ranged from 40 to 70 nM; peak values were between 150 and 300 nM.

The α2-adrenergic receptor antagonist yohimbine inhibited the Ca^{2+} increase with a K_i value of 3.8 nM (Table I), and the α2-selective adrenergic receptor antagonist BE 2254 had a K_i value of 22.4 nM (Table I) which matches its K_i for inhibition of [3H]yohimbine binding to platelet α2-adrenergic receptors (16). Thus, the epinephrine-stimulated Ca^{2+} increase appears to be mediated by α2-adrenergic receptors. As α2-adrenergic receptors can be subdivided into α2A- and α2B-subtypes, we...
tested three compounds that can discriminate between them. Prazosin in concentrations up to 100 nM did not antagonize the Ca\(^{2+}\) increase; higher concentrations could not be tested because it fluoresced (data not shown); oxymetazoline was a pure antagonist in this system with a \(K_i\) value of 8.5 nM (Table I) which matches that described at \(\alpha_2\)-adrenergic receptors (17). ARC 239 had a \(K_i\) value of 0.68 \(\mu\)M (Table I) which also matches that at \(\alpha_2\)-adrenergic receptors (17). The \(K_i\) value for the inhibition of forskolin-stimulated cAMP generation by epinephrine (2.3 ± 0.33 \(\mu\)M; \(n = 3\)) suggested that this response is also mediated by \(\alpha_2\)-adrenergic receptors.

The order of potency for several \(\alpha\)-adrenergic receptor agonists for increasing intracellular Ca\(^{2+}\) was UK 14,304 > epinephrine > oxymetazoline (Table II). This is identical to the order of potency for inhibition of forskolin-stimulated cAMP generation in these cells in the presence of 1 \(\mu\)M propranolol (Table II). In both assays all three agonists had similar maximal effects (data not shown). Oxymetazoline and clonidine did not have any agonistic effects in the calcium assay and were partial agonists for inhibiting cAMP generation (Table II).

Increases in intracellular Ca\(^{2+}\) can be due either to mobilization from intracellular stores or to influx of extracellular Ca\(^{2+}\). Chelating extracellular Ca\(^{2+}\) with 5 mM EGTA did not blunt the increase in intracellular Ca\(^{2+}\) by epinephrine (Fig. 2), demonstrating that extracellular Ca\(^{2+}\) is not required. Further additions of EGTA or EDTA did not lower Fura-2 fluorescence, demonstrating that extracellular Ca\(^{2+}\) was completely chelated by 5 mM EGTA. Thus stimulation of \(\alpha_2\)-adrenergic receptors mobilizes Ca\(^{2+}\) from intracellular stores.

Because \(\alpha_2\)-adrenergic receptor stimulation both increases Ca\(^{2+}\) and decreases cAMP, we asked whether one of the two events mediates the other. Increasing the cAMP levels by 20 \(\mu\)M forskolin or addition of 10 \(\mu\)M 8-bromo-cAMP did not reduce the epinephrine-induced Ca\(^{2+}\) mobilization (Table III). As our HEL cells contain \(\beta\)-adrenergic receptors which stimulate adenylate cyclase, epinephrine (acting at both \(\alpha_2\)- and \(\beta\)-adrenergic receptors) inhibited forskolin-stimulated cAMP generation in the presence, but not in the absence, of propranolol (Fig. 3, left). However, epinephrine increased intracellular Ca\(^{2+}\) similarly whether or not propranolol was present (Fig. 3, right) and thus, whether or not cAMP was decreased. We conclude that the Ca\(^{2+}\) mobilization is not caused by decreased cAMP levels. We also tested whether elevated Ca\(^{2+}\) can alter cAMP levels. The ionophore ionomycin (30 \(nM\)) elevated intracellular Ca\(^{2+}\) to a peak level somewhat higher than that achieved by 10 \(\mu\)M epinephrine but did not alter basal or forskolin-stimulated cAMP generation (data not shown).

\(\alpha_2\)-Adrenergic receptors may couple to Na\(^+\)/H\(^+\) exchange, which is blocked by placing cells in a sodium free buffer (2, 18). Removal of extracellular Na\(^+\) did not decrease the epinephrine-promoted Ca\(^{2+}\) mobilization (Table III). Moreover, the ionophore monensin, which exchanges Na\(^+\) for H\(^+\), did not alter intracellular Ca\(^{2+}\) (not shown). Thus, the Ca\(^{2+}\) mobilization is not secondary to Na\(^+\)/H\(^+\) exchange.

We then asked whether the epinephrine-stimulated Ca\(^{2+}\) increase is mediated by a G-protein. Pretreatment of cells with pertussis toxin (known to inactivate several G-proteins) completely blocked both the \(\alpha_2\)-adrenergic receptor-mediated increase in Ca\(^{2+}\) and inhibition of cAMP accumulation (Fig. 4). Antibodies against Go, (18) did not react with HEL cell membranes in Western blots (Fig. 5). As this antibody can detect as little as 20 ng of Go, Go is either absent from HEL cells or present as less than 0.01% of the membrane protein. In contrast, an antibody able to recognize various forms of Gi (14) cross-reacted with a 41-kDa protein in HEL membranes (Fig. 5).

Stimulation of \(\alpha_2\)-adrenergic receptors in platelets releases arachidonic acid which is metabolized to thromboxane A\(_2\) which increases intracellular Ca\(^{2+}\) (19). The production of
thromboxane can be inhibited by preincubation with the cyclooxygenase inhibitors aspirin or indomethacin. Thus epinephrine does not elevate cytosolic Ca2+ in aspirin-pretreated platelets (20-23). In HEL cells, however, pretreatment with aspirin or indomethacin did not reduce epinephrine-stimulated Ca2+ increases and inhibition of cAMP generation are not involved either (Table IV). Moreover, adding 10 μM aspirin or indomethacin did not stimulate cGMP accumulation in HEL cells (basal: 0.10 ± 0.01 fmol/107 cells; n = 3). We also measured cGMP levels in HEL cells, as increases in intracellular cGMP accompany increases in intracellular Ca2+ in some systems (24-26). However, epinephrine (10 μM) did not stimulate cGMP accumulation in HEL cells (basal: 0.48 ± 0.18, epinephrine: 0.46 ± 0.10 fmol/107 cells; n = 3).

**DISCUSSION**

Stimulation of α2-adrenergic receptors inhibits adenylyl cyclase in every system studied, but additionally these receptors can couple to other second messengers in some systems. Our data demonstrate that α2-adrenergic receptors on HEL cells couple to Ca2+ mobilization by a mechanism independent of inhibition of adenylyl cyclase.

Like inhibition of adenylyl cyclase, the epinephrine-promoted Ca2+ increase is mediated by an α2-adrenergic receptor, as shown by the Ki values of subtype selective α-adrenergic antagonists and the order of potency of agonists (Tables I and II). The existence of more than one type of α2-adrenergic receptors has been suggested by both pharmacological data and the presence of multiple receptor genes (27, 28). Our pharmacological data suggest that both the epinephrine-stimulated Ca2+ increases and inhibition of cAMP generation are mediated primarily by the α2A-subtype. This is not surprising...
as HEL cells are related to platelets, which have a homogeneous population of $\alpha_2$-adrenergic receptors.

The agonists epinephrine, UK14,304, and moxonidine had similar maximal effects for increasing intracellular $Ca^{2+}$ and inhibiting cAMP generation. The partial agonists clonidine and oxymetazoline were only 30–40% as efficacious as epinephrine in inhibiting cAMP generation and were not able to elevate $Ca^{2+}$. Thus, a greater intrinsic activity seems to be necessary for increasing $Ca^{2+}$ than for inhibiting cAMP generation. This may also explain the 7-fold higher concentration of moxonidine needed to elevate $Ca^{2+}$ than to inhibit cAMP generation; perhaps moxonidine is not a full agonist.

$\alpha_2$-Adrenergic receptors have been previously linked to elevation of intracellular $Ca^{2+}$ in two ways: causing an influx of extracellular $Ca^{2+}$ and indirectly mobilizing intracellular $Ca^{2+}$ via release of arachidonic acid metabolites. As discussed below, our data shows that $Ca^{2+}$ increases in HEL cells occur by a different mechanism.

$\alpha_2$-Adrenergic receptor-stimulated influx of $Ca^{2+}$ has been postulated in smooth muscle and platelets. Constriction of vascular smooth muscle via postsynaptic $\alpha_2$-adrenergic receptors requires extracellular $Ca^{2+}$ and is blocked by calcium entry blockers (29). In platelets, coupling of $\alpha_2$-adrenergic receptors to $Ca^{2+}$ influx has been suggested by some data obtained using aequorin (30) but not other data (obtained using Fura-2 (23), Quin2 (20, 21) or Indo-1 (22)). However, the increase in intracellular $Ca^{2+}$ we observe in HEL cells does not require extracellular $Ca^{2+}$.

In platelets, epinephrine causes release of arachidonic acid, which is metabolized to thromboxane $A_2$, which mobilizes intraplatelet $Ca^{2+}$ via IP$_3$ production. Thromboxane production in platelets is blocked by the cyclooxygenase inhibitors aspirin or indomethacin. In contrast, epinephrine-stimulated $Ca^{2+}$ mobilization in HEL cells was not blocked by cyclooxygenase or lipoxygenase inhibitors and was not mimicked by the thromboxane $A_2$ analogue U46619. Epinephrine-stimulated arachidonic acid release from platelets appears to be mediated by activation of $Na^+-H^+$ exchange, which requires the presence of extracellular $Na^+$ (3). In contrast, $Ca^{2+}$ mobilization in HEL cells did not require extracellular $Na^+$. Thus, mobilization of $Ca^{2+}$ in HEL cells does not depend upon thromboxane generation and does not require $Na^+-H^+$ exchange.

Many receptors are coupled to a mobilization of intracellular $Ca^{2+}$ by activating phospholipase C, leading to generation of IP$_3$ which releases $Ca^{2+}$ from intracellular sites such as the endoplasmic reticulum. However, our data suggest that this is probably not the mechanism of $\alpha_2$-adrenergic receptor-mediated $Ca^{2+}$ mobilization in HEL cells. Using two methods, we did not detect any increase in IP$_3$ by epinephrine at early time points matching the $Ca^{2+}$ increase (Fig. 6, Table IV). On the other hand, we detected an increased inositol phosphate generation after a 5-min incubation with epinephrine, i.e. long after the $Ca^{2+}$ levels had returned to base line. However, this was not blocked by neomycin (which inhibits inositol phosphate generation in some other systems (31)). Moreover, neuropeptide $Y$ increases intracellular $Ca^{2+}$ in HEL cells to a level twice as high as does epinephrine but increases inositol phosphate generation at 5 min only half as much as does epinephrine (12). Thus, we cannot rule out the possibility that $Ca^{2+}$ is mobilized by a small pool or unusual isomer of inositol phosphates, but we suggest that an entirely different mechanism accounts for the $Ca^{2+}$ mobilization in HEL cells.

An inositol phosphate-independent mechanism for $Ca^{2+}$ mo-
α2-Adrenergic Mobilization of Ca^{2+}

Mobilization has also been suggested by other data. For example, α1-adrenergic receptors on BC3H-1 muscle cells mobilize intracellular Ca^{2+} maximally by 30 s, but increase inositol phosphates only much later (32, 33).

The α2-adrenergic receptor-mediated Ca^{2+} mobilization is inhibited by pertussis toxin (Fig. 4). Pertussis toxin ADP-ribosylates and thus inactivates several G proteins including G_i (mediating inhibition of adenylate cyclase) and G_o (thought to mediate inhibition of a voltage-sensitive Ca^{2+} channel) (34). The Western blots demonstrated that the HEL cells do not contain G_o (Fig. 5). Thus, the mobilization of Ca^{2+}, like the inhibition of adenylate cyclase, may be mediated by G_o. However, multiple forms of G_i have been identified by cloning and sequencing studies (35), and HEL cells contain at least two forms of G_o: α_1D and α_2 (36). The present data cannot discriminate whether or not the same G protein mediates α2-adrenergic stimulation of Ca^{2+} mobilization and inhibition of adenylate cyclase.

Ca^{2+} mobilization was not altered by forskolin or 8-bromocAMP. Moreover, although epinephrine-mediated inhibition of cAMP accumulation required blockade of β-adrenergic receptors with propranolol, Ca^{2+} mobilization occurred in both the presence and absence of propranolol. Ca^{2+} elevation by ionomycin did not alter cAMP generation. Thus, inhibition of cAMP generation and mobilization of Ca^{2+} occur independently.

Acknowledgments—We thank Dennis Smith and Steve Carter for technical help, Karin Norgard for early experiments, Laurence Brunton for use of the fluorometer, Thalia Papayannopoulou for providing HEL cells, and Paul Insel for helpful comments.

REFERENCES