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Eosinophils play a key role in the pathogenesis of asthma and other allergic inflammatory diseases. We have previously shown that treatment of eosinophils with lidocaine preferentially inhibits IL-5-induced survival. This inhibition cannot be overcome by increasing concentrations of IL-5 and is not due to the blocking of Na+ channels by lidocaine. Here we report that one class of K+ channel blockers, the sulfonyleureas, inhibits eosinophil survival in a manner similar to lidocaine. The sulfonyleurea glyburide inhibits eosinophil survival even at high concentrations of IL-5. In contrast, increasing concentrations of IL-3 or granulocyte-macrophage CSF overcome glyburide inhibition. Glyburide also blocks cytokine-induced eosinophil superoxide production. Similar results were seen with the sulfonyleureas tolvatamide and glipizide. Interestingly, the effects of glyburide are not antagonized by the ATP-sensitive K+ channel openers cromakalim, pinacidil, or diazoxide. Although Scatchard analysis of [3H]glyburide binding to eosinophil membranes indicated that the high affinity sulfonyleurea receptor (SUR1) is not present on eosinophils, human eosinophils do express mRNA homologous to the sulfonyleurea receptor family, in keeping with the presence of a sulfonyleurea receptor. Finally, coculture of eosinophils with combinations of glyburide, lidocaine, and dexamethasone resulted in synergistic inhibition of cytokine-mediated eosinophil survival and superoxide production. These results have intriguing clinical implications for the treatment of eosinophil-associated diseases.

Human asthma is characterized by reversible bronchial hyperreactivity and infiltration of inflammatory cells, especially eosinophils, into the lung (1, 2). In recent years it has become obvious that treating the underlying inflammation is critical to maintaining good airway function (3). The most potent agents currently used for controlling inflammation are the glucocorticoids. However, the adverse effects of long term treatment with oral glucocorticoids have stimulated efforts to identify effective anti-inflammatory substitutes (4, 5). We have previously reported the clinical efficacy of the topical anesthetic lidocaine in the treatment of asthma in an open label trial (6). Treatment of glucocorticoid-dependent patients with nebulized lidocaine allowed for the complete elimination of steroid treatment in 13 of 20 patients, although exacerbations still required oral steroids. This study suggests that lidocaine functions as an anti-inflammatory agent and is thus able to replace long term oral steroid treatment.

Lidocaine, like the glucocorticoids, may partially exert anti-inflammatory effects by directly inhibiting eosinophil survival and activation in the lung. In the absence of exogenous cytokines, such as IL-5, IL-3, or GM-CSF, eosinophils undergo apoptosis (7–9). This mode of cell death with consequent engulfment by macrophages appears to be critical for preventing the release of toxic eosinophil granule proteins into sensitive tissues, such as the lung (7–11). If, however, cytokines are present (as they are in bronchial lavage fluid from patients with asthma (12)) eosinophil survival is prolonged, granule proteins are released (13), and eosinophil apoptosis is substantially delayed (12). We reported previously that lidocaine treatment of eosinophils in vitro ablates cytokine-induced survival in a manner similar to dexamethasone (14–16). Other topical anesthetics tested blocked eosinophil survival as well, but their potencies in the survival assay did not correlate with their potencies as anesthetics (17). Additionally, Na+ channel blockers that should mimic the anesthetic effect of lidocaine had no effect on cytokine-stimulated eosinophil survival in vitro (17, 18). These results support the hypothesis that the effect of lidocaine on eosinophil survival is a novel function of the drug.

Several reports in the literature indicate that lidocaine at high concentrations blocks K+ channels (19–21). Using three classes of K+ channel blockers, we investigated the effect of K+ channel regulation on eosinophil survival. Here we report that the sulfonyleureas, one class of K+ channel blockers, inhibit eosinophil survival and activation in vitro.

Materials and Methods

Materials

HIT T15 cells were obtained from American Type Culture Collection (Rockville, MD). Human rIL-5 was a gift from Schering Plough (Kenilworth, NJ). IL-3 and GM-CSF were purchased from R&D Systems (Minneapolis, MN). Tolbutamide, glipizide, apamin, tetraethylammonium chloride (TEA), diazoxide, and pinacidil were purchased from Research Biochemicals International (Natick, MA). Unless otherwise indicated, all other materials were purchased from Sigma (St. Louis, MO).

Eosinophil purification

Eosinophils were purified from normal human subjects and patients, usually allergic, with slightly elevated numbers of eosinophils using a magnetic bead separation protocol previously described (22, 23). Briefly, blood was layered on Percoll (density = 1.085 g/ml) and centrifuged at 900 × g to remove PBMC and hypodense (activated) eosinophils. Erythrocytes
were lysed by osmotic shock, and neutrophils were removed from the eosinophils by separation using a steel wool column (type CS) and anti-CD16-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA). Eosinophil purity exceeded 95% as determined by Randolph’s stain. The primary contaminating cells were neutrophils.

**Survival assays**

Purified eosinophils were washed with HybriCare medium (American Type Culture Collection) supplemented with 2 mM glutamine, 30 μg/ml gentamicin sulfate, and 2.5 to 10% α calf serum (HyClone, Logan, UT). Eosinophils were resuspended at 0.5 × 10^6 cells/ml in supplemented HybriCare and were incubated with drugs and cytokines in 96-well plates (Costar, Cambridge, MA) as indicated in the text. Drugs that were not directly soluble in media were first dissolved in DMSO and diluted to the appropriate concentrations in media. Unless indicated, the final DMSO concentration in the well never exceeded 0.5%, which was shown in control experiments to have no effect on eosinophil survival. The final concentration of eosinophils was 5 × 10^4/well in a total volume of 200 μl. Plates were incubated at 37°C in 5% CO2 for 4 days. At that time, each well was transferred into 12-× 75-mm polystyrene tubes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ), and 200 μl of 0.1 M propidium iodide (PI; final concentration, 0.5 μg/ml) were added to each tube to stain dead cells. Stained cells were analyzed using a FACSscan flow cytometer and PC LYSIS software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Eosinophil survival is plotted as relative percent survival (percentage of the control) according to the following equation: relative eosinophil survival = (% survival/percent survival at maximum cytokine concentration) × 100. Eosinophils cultured with IL-5 (1000 pg/ml; = 22 pM) usually showed >90% survival, whereas cells cultured in control medium usually had <30% spontaneous survival.

**Apoptosis assay**

Eosinophil apoptosis was assayed using flow cytometry and the DNA-binding fluorophores Hoechst 33342 (Molecular Probes, Eugene, OR) and PI as previously described (24, 25). Following 48 h of stimulation, eosinophils were stained with 10 μM Hoechst and 32 μM PI and analyzed on a Becton Dickinson FACS Vantage flow cytometer (San Jose, CA) with UV laser excitation at 357 nm. Dead cells fluoresced red and blue, live cells had low intensity blue fluorescence only, and apoptotic cells had high intensity blue fluorescence.

**Superoxide assay**

Ninety-six-well plates were blocked with 50 μl of 1% human serum albumin for 2 h at 37°C. Before adding cells, blocked wells were washed twice with 0.05% of saline. Purified eosinophils were washed with HBSS medium (10 mM HEPES, pH 7.4) and resuspended to 5 × 10^5 cells/ml in 2 ml of saline. Eosinophils were added to 0.1 ml of 50 mM 3-iodo-2-nitrobenzamide (Molecular Probes, Eugene, OR) and were pretreated with drugs and cytokines in 96-well plates (Costar, Cambridge, MA) every 15 to 30 min for 2 h. Conversion of absorbance to nanomoles of cytochrome c reduced per 1 × 10^6 eosinophils used the following equation: 19.1(1 - absorbance − absorbance in blanks)/0.05 = nmol cytochrome c reduced/10^6 cells (23).

**Glyburide binding assay**

Cell membranes were prepared as previously described (26), and 20 μg of membrane protein was incubated in 0.1 ml of 50 mM 3-(N-morpholino)propanesulfonic acid, pH 7.4, with 0.05–50 nM [3H]glyburide (Amersham, Arlington Heights, IL) for 2 h at room temperature. Bound radioligand was separated from free using a Skatron cell harvester (Skatron Instruments, Sterling, VA) with double glass filters. The filters were dried and counted in 2 ml of scintillation fluid using a beta counter. Specific picomoles of [3H]glyburide bound per milligram of protein were determined by subtracting nonspecific binding in the presence of excess unlabeled glyburide.

**RNase protection assay**

Biotinylated probes for human actin or the NBF2 region of human sulfonylurea receptor (SUR) (27) were made with the BrightStar BiOTINscript Kit (Ambion, Austin, TX). Guanidine thiocyanate lysates of purified human eosinophils were prepared and analyzed with the Direct Protect Lysate Ribonuclease Protection Assay Kit (Ambion). Briefly, the lysates were incubated with biotinylated probes and treated with RNase to degrade any remaining single-stranded probe. After the RNase was inactivated with sarcosyl and proteinase K, the RNase-protected fragments were precipitated, resuspended in loading buffer, and separated on a 10% polyacrylamide gel. The gel was transferred to a positively charged membrane (Ambion), and hybridized biotinylated probe was detected using the BrightStar BioDetect Kit (Ambion) and Kodak autoradiographic film (Eastman Kodak, Rochester, NY).

**Statistical analysis**

Statistical analyses were performed using two-tailed, paired Student’s t test. Statistical analysis of dose-dependent glyburide apoptosis induction (data not shown) was calculated using the Spearman rank correlation coefficient.

**Results**

**Effects of K**+ channel blockers on human eosinophil survival**

To test the hypothesis that K+ channels are involved in eosinophil survival, we compared the activities of three different K+ channel blockers (glyburide, TEA, and apamin) with the activity of lidocaine on IL-5-stimulated eosinophils. Glyburide is a blocker of ATP-sensitive K+ channels, TEA blocks K+ channels, and is a nicotinic cholinergic receptor antagonist, and apamin blocks Ca2+-activated K+ channels (28, 29). As shown in Figure 1, TEA and apamin had no significant effect on IL-5-induced eosinophil survival. Only glyburide (1 × 10^-4 M) inhibited IL-5-mediated eosinophil survival as effectively as lidocaine. To determine whether this was a dose-response effect, doses of glyburide between 10^-5 and 10^-4 M were tested. As shown in Figure 2, glyburide exhibited a dose-response effect, which was especially evident at lower IL-5 concentrations. However, only the inhibition mediated by 10^-4 M glyburide was statistically significant.

One of the characteristics of lidocaine inhibition of eosinophil survival is a relative specificity for IL-5 (17). To determine whether glyburide exhibits a similar preference, we examined the ability of glyburide to inhibit eosinophil survival mediated by IL-3 and GM-CSF. As shown in Figure 3, eosinophil survival was inhibited by glyburide at low IL-3 and GM-CSF concentrations, but the inhibition was completely overcome by increased concentrations of these cytokines. This pattern of inhibition was essentially identical with that seen with lidocaine (compare left and right columns of Fig. 3). Glyburide and lidocaine both preferentially inhibit IL-5-induced survival compared with IL-3- or GM-CSF-mediated survival, and the inhibition could not be overcome by increased concentrations of IL-5 (up to 10,000 pg/ml; data not shown). Neither lidocaine nor glyburide mediated a general toxic effect on the cells, since eosinophils stimulated with IL-3 or GM-CSF survived in the presence of the drugs. Additionally, the decreased eosinophil survival seen in the glyburide-treated cells correlated with increased apoptosis, as detected by staining cells with Hoechst 33342 and PI (data not shown). This effect was statistically significant (p < 0.05) and dose dependent (r = 1.0; p < 0.05). Additionally, the concentrations of glyburide required to increase apoptosis were essentially identical with the concentrations required to inhibit eosinophil survival.

Glyburide, also called glybenclamide, belongs to the sulfonylurea family of compounds. Sulfonylurea blocks ATP-sensitive K+ channels by binding to an accessory regulating receptor, the SUR (30). If glyburide inhibits eosinophil survival by blocking an ATP-sensitive K+ channel, then other sulfonylureas should have similar activities. To test this, we examined the effects of two other sulfonylureas: tolbutamide and glipizide. As shown in Figure 4, tolbutamide and glipizide inhibited eosinophil survival as well as did glyburide. High concentrations (1000 pg/ml) of IL-3 or GM-CSF...
overcame the inhibitory effects of the sulfonylureas, whereas eosinophils treated with 1000 pg/ml IL-5 in the presence of glyburide, tolbutamide, or glipizide showed a 20 to 30% average decrease in survival. Thus, glyburide is representative of the general effectiveness of sulfonylureas in inhibiting eosinophil survival.

**K⁺ channel openers rescue stressed eosinophils**

If blocking an ATP-sensitive K⁺ channel results in inhibition of eosinophil survival, then an ATP-sensitive K⁺ channel opener should block the effects of glyburide and/or potentiate survival (31). To test this, we examined the effects of the ATP-sensitive K⁺ channel opener cromakalim on IL-5-mediated eosinophil survival.

We were unable to inhibit the effects of glyburide by cromakalim pretreatment despite repeated efforts (n = 8; data not shown). However, we observed the potentiation of eosinophil survival in the presence of IL-5. As shown in Figure 5, the IL-5-mediated survival in these experiments was markedly less than usual; eosinophil absolute survival is normally >90% after 4-day stimulation with 1000 pg/ml IL-5. The reason for the low survival in these experiments was a high (1%) final concentration of DMSO in the medium from preparation of the cromakalim stock. However, this decreased survival response to IL-5 in the presence of 1% DMSO allowed us to observe the phenomenon of cromakalim “rescue” of eosinophils. As shown in Figure 5, this rescue was dramatic and restored the survival of IL-5-treated eosinophils to normal. This rescue was also observed when eosinophils were treated with the K⁺ channel openers pinacidil and diazoxide and was a dose-dependent effect (data not shown).

**Glyburide inhibits human eosinophil superoxide production**

Eosinophil activation and degranulation, not their mere presence, are probably critical in initiating the tissue damage associated with eosinophilic inflammation (1). Activation of eosinophils in vitro can be detected by measuring superoxide production in response to stimuli. We have previously shown that lidocaine significantly inhibits cytokine-mediated eosinophil superoxide production (17). To determine whether glyburide also inhibits eosinophil superoxide production, we cultured eosinophils with IL-5, IL-3, or GM-CSF in the presence of glyburide. As shown in Figure 6, all three cytokines stimulated superoxide production by eosinophils. However, superoxide production initiated by the cytokines was dramatically inhibited by glyburide, and excess cytokine did not overcome this inhibition. Interestingly, no preferential inhibition of IL-5 stimulation was seen in the superoxide assay compared to that in the survival assay. Instead, glyburide inhibited superoxide generation induced by all cytokines, including that generated by platelet-activating factor (data not shown). As in the survival assay, pretreatment of eosinophils with cromakalim did not block the glyburide effect under normal (low DMSO) conditions (data not shown).
Lidocaine, glyburide, and dexamethasone synergize to inhibit eosinophil survival and activation

Lidocaine and glyburide directly inhibit cytokine-mediated eosinophil survival and activation, probably through mechanisms distinct from those of dexamethasone (16). This follows because glucocorticoid inhibition of IL-5-stimulated survival is overcome at a concentration of 1000 pg/ml IL-5, whereas this is not the case with lidocaine or glyburide. Thus, these drugs may have additive or even synergistic effects when used in combination. To test this, we incubated eosinophils with IL-5 in the presence of lidocaine, dexamethasone, glyburide, or combinations of these drugs. As shown in Table I, eosinophils treated with lidocaine, dexamethasone, glyburide, or combinations of these drugs. As shown in Table I, eosinophils treated with IL-5 in the presence of lidocaine, dexamethasone, glyburide, or combinations of these drugs. As shown in Table I, eosinophils treated with IL-5 in the presence of lidocaine, dexamethasone, glyburide, or combinations of these drugs.

Eosinophils express a SUR

The effects of the sulfonylureas suggest that a SUR is present on eosinophils. To detect message for a SUR family member in eosinophil lysates, we used a biotinylated RNA probe for the conserved NBF2 region of SUR1 (27) in a ribonuclease protection assay (RPA). The NBF2 region is conserved in both SUR1 and SUR2, and is presumably common to the entire family of SUR proteins (32). Binding of the SUR probe should result in a protected mRNA fragment of 80 bp. As shown in Figure 8, eosinophil lysates from three separate donors expressed mRNA that hybridized with the SUR probe (lanes 6, 8, and 10), indicating that the eosinophils are actively transcribing a gene that is homologous to the NBF2 region of human SUR1.

To characterize this putative SUR on human eosinophils, we performed binding assays using [3H]glyburide as a ligand. As a positive control we used the HIT T15 hamster cell line, previously shown to express a functional SUR1 (27, 33). Scatchard analysis of equilibrium binding data revealed high affinity ($K_d = 0.42$ nM) binding sites on HIT cell membranes (data not shown), in agreement with published values. However, in three separate experiments, human eosinophil membranes did not show specific [3H]glyburide binding, and in the one experiment showing specific binding, Scatchard analyses of equilibrium binding data showed a poor correlation (coefficient $<0.5$) between the bound/free ratio and picomoles of ligand bound (data not shown). Additionally, independent photoaffinity labeling experiments performed on prepared eosinophil membranes failed to show surface labeling with glyburide (data not shown; L. Aguilar-Bryan, unpublished observations). These data indicate that high affinity SUR1 is not present on human eosinophils. This is consistent with the concentrations of glyburide necessary to exert an effect on eosinophils. The inability to detect specific binding in this assay suggests that the SUR on eosinophils has a low affinity for glyburide, similar to SUR2.
Discussion

Apoptosis of lung eosinophils is correlated with the resolution of airway inflammation in asthma (10, 11, 34). Glucocorticoid therapy presumably is effective in part by directly inhibiting cytokine-mediated eosinophil survival, resulting in eosinophil apoptosis and clearing by macrophages (16). Here we report that sulfonyleureas, including glyburide, tolbutamide, and glipizide, mimic glucocorticoids by inhibiting cytokine-mediated survival of eosinophils obtained from both normal and allergic patients. However, the sulfonyleureas differ from the glucocorticoids because the effects of the sulfonyleureas, unlike those of the glucocorticoids, cannot be overcome with high concentrations of IL-5, a key mediator of eosinophil survival and activation in vitro and in vivo (12, 35, 36). This preferential inhibition of IL-5-induced signals is also observed when eosinophils are treated with lidocaine (14, 17). The ability of glyburide and lidocaine to synergize with dexamethasone in the survival assay suggests that the actions of the drugs are complimentary and work through independent mechanisms. One can speculate that glyburide and lidocaine target a channel(s), whereas glucocorticoids work at the nuclear level to alter gene transcription (37).

To damage the airway epithelium and promote bronchial hyperreactivity, eosinophils must not only be present, but also activated (38, 39). Glyburide and lidocaine are effective at blocking cytokine-induced superoxide production. Interestingly, the drugs show no specificity for IL-5 stimulation (as they do in the survival assay), but inhibit equally well superoxide induced by IL-3 and GM-CSF. The combination of lidocaine and glyburide in the superoxide assay resulted in synergistic inhibition of eosinophil activation, suggesting that in this assay lidocaine and glyburide have unique mechanisms of action. It is interesting that glucocorticoids do not inhibit eosinophil superoxide production, suggesting that glyburide (and lidocaine) may be more effective in the prevention of eosinophil-mediated tissue damage.

Similar functions of glyburide and lidocaine suggest that the sulfonyleureas and lidocaine may be working through similar mechanisms. Our proposed model is that lidocaine exerts its effects by directly blocking a K⁺ channel, comparable to its blocking of Na⁺ channels (18). Similarly, sulfonyleureas block ATP-sensitive K⁺ channels by binding to the associated SUR on the cell surface. Thus, both drugs may exert their effects by blocking K⁺ channels, but through different mechanisms. This model is consistent with our results showing that lidocaine and glyburide have the same effects in biologic assays using eosinophils. However, our results showing synergy of glyburide and lidocaine are more difficult to explain in light of this model, since synergistic compounds typically act through different mechanisms. It is quite possible that glyburide and lidocaine, in addition to the mechanisms stated above, exert their effects on eosinophils through alternative means as well. This is evidenced by the ability of glyburide, but not lidocaine (17), to block platelet-activating factor-induced superoxide production. Thus, the similar biologic effects of lidocaine and glyburide could be explained by their shared targeting of a K⁺ channel.
channel, whereas the ability of the two drugs to synergize could be attributed to the individual effects of each drug on other channels or signaling receptors.

It is intriguing that glyburide shows different specificities in the eosinophil survival and activation assays. High concentrations of GM-CSF and IL-3, unlike IL-5, can overcome glyburide inhibition of survival, but not inhibition of superoxide generation. Although the receptors for GM-CSF, IL-3, and IL-5 share a common β-chain that is thought to be responsible for signal transduction, several reports have indicated a signaling role for the specific α-chains of the receptors (40–44). Thus, one could postulate that the signaling events responsible for prolonging eosinophil survival are influenced by the α-chain, whereas the signals required for superoxide production depend primarily on β-chain activation. This hypothesis could explain why IL-5-induced survival is more sensitive to glyburide (and lidocaine) than that induced by IL-3 or GM-CSF, and why superoxide production initiated by all three cytokines is equally well inhibited. Support for this view comes from a recent report describing the biologic activity of an IL-5 protein in which an important β-chain contact site contained a charge reversal, thus affecting β-chain activation and subsequent signal transduction (45). The mutant IL-5 still induced eosinophil survival, but did not induce activation (as measured by adhesion) and, in fact, functioned as a specific antagonist in that assay.

A SUR family has recently been identified, and members of the family associate with and regulate the inwardly rectifying K+ channel, Kir6.2 (32). The two identified members, SUR1 and SUR2, are structurally related but functionally distinct, in that the SUR2/Kir6.2 combination is less sensitive to glyburide than is the SUR1/Kir6.2 complex. Based on the concentration of glyburide necessary to inhibit eosinophil survival in our experiments (10−4 M), we speculate that glyburide is not acting through the classic SUR1/Kir6.2 complex. However, the results from our binding and RPA studies indicate that the affinity of the eosinophil SUR for glyburide is lower than that of the SUR1/Kir6.2 complex, and why superoxide production initiated by all three cytokines is equally well inhibited. Support for this view comes from a recent report describing the biologic activity of an IL-5 protein in which an important β-chain contact site contained a charge reversal, thus affecting β-chain activation and subsequent signal transduction (45). The mutant IL-5 still induced eosinophil survival, but did not induce activation (as measured by adhesion) and, in fact, functioned as a specific antagonist in that assay.
much less than that of SUR1. This is consistent with the eosinophil glyburide target being SUR2-like, as the affinity of SUR2 for glyburide hampers detectable surface binding (L. Aguilar-Bryan, unpublished observations).

Although we could not detect SUR1 on eosinophil membranes, the presence of a SUR homologous transcript is worth emphasizing. In the absence of specific Abs to confirm the existence of a SUR, then we should be able to reverse that inhibition with an ATP-sensitive K⁺ channel opener, such as cromakalim (31). Interestingly, the ATP-sensitive K⁺ channel opener cromakalim has been tested in clinical trials as an oral asthma drug (47–50). If glyburide is exerting its effects on eosinophil survival and activation by binding to a conventional SUR, then we should be able to reverse its effect with a SUR. Other ion channels, such as the cystic fibrosis transmembrane regulator, are inhibited by the sulfonylureas (51). We are confident that the eosinophil SUR is not SUR1, based on both its lack of responsiveness to cromakalim (under low DMSO conditions) and the high (10⁻⁴ M) concentration of glyburide required to inhibit eosinophil survival. Thus, eosinophils may express SUR2 or a novel SUR family member with distinct characteristics.

The observations presented here have intriguing clinical implications for the treatment of asthma and other eosinophil-associated diseases. The results suggest that ion channels on eosinophils, especially ATP-sensitive K⁺ channels, may be targets for future drug development. Achieving a concentration of 10⁻³ M glyburide in an asthmatic lung is most likely possible, but may precipitate unwanted side effects. The synergy of the sulfonylureas with glucocorticoids and lidocaine could allow the use of lower concentrations if necessary. Also, the risk of adverse reactions resulting from sulfonylurea treatment would be minimized by local (rather than systemic) application. Because lidocaine has been shown to be effective in an open label clinical trial (6), the sulfonylureas may similarly represent a potential class of anti-inflammatory asthma drugs for the treatment of asthma and eosinophil-associated diseases.

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