Glycine-gated chloride channels in neutrophils attenuate calcium influx and superoxide production

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ABSTRACT **Recently, it was demonstrated that liver injury and TNF-α production as a result of endotoxin (lipopolysaccharide, LPS) were attenuated by feeding animals a diet enriched with glycine. This phenomenon was shown to be a result of, at least in part, activation of a chloride channel in Kupffer cells by glycine, which hyperpolarizes the cell membrane and blunts increases in intracellular calcium concentrations ($[Ca^{2+}]_i$) similar to its action in the neuron. It is well known that hepatotoxicity due to LPS has a neutrophil-mediated component and that activation of neutrophils is dependent on increases in $[Ca^{2+}]_i$. Therefore, the purpose of this study was to determine if glycine affected agonist-induced increases in $[Ca^{2+}]_i$ in rat neutrophils. The effect of glycine on increases in $[Ca^{2+}]_i$ elicited either by the bacterial-derived peptide formyl-methionine-leucine–phenylalanine (FMLP) or LPS was studied in individual neutrophils using Fura-2 and fluorescence microscopy. Both FMLP and LPS caused dose-dependent increases in $[Ca^{2+}]_i$, which were maximal at 1 μM FMLP and 100 μg/ml LPS, respectively. LPS increased intracellular calcium in the presence and absence of extracellular calcium. Glycine blunted increases in $[Ca^{2+}]_i$ in a dose-dependent manner with an IC_{50} of ~0.3 mM, values only slightly higher than plasma levels. Glycine was unable to prevent agonist-induced increases in $[Ca^{2+}]_i$ in chloride-free buffer. Moreover, strychnine (1 μM), an antagonist of the glycine-gated chloride channel in the central nervous system, reversed the effects of glycine (1 mM) on FMLP- or LPS-stimulated increases in $[Ca^{2+}]_i$. To provide hard evidence for a glycine-gated chloride channel in the neutrophil, the effect of glycine on radioactive chloride uptake was determined. Glycine caused a dose-dependent increase in chloride uptake into neutrophils with an ED_{50} of ~0.4 mM, an effect also prevented by 1 μM strychnine. Glycine also significantly reduced the production of superoxide anion from FMLP-stimulated neutrophils. Taken together, these data provide clear evidence that neutrophils contain a glycine-gated chloride channel that can attenuate increases in $[Ca^{2+}]_i$ and diminish oxidant production by this important leukocyte.—Wheeler, M., Stachlewitz, R. F., Yamashina, S., Ikejima, K., Morrow, A. L., and Thurman, R. G. Glycine-gated chloride channels in neutrophils attenuate calcium influx and superoxide production. *FASEB J.* 14, 476–484 (2000)**

Key Words: glycine · strychnine · intracellular calcium · lipopolysaccharide · formyl-methionine-leucine-phenylalanine

A DIET ENRICHED with the nonessential amino acid glycine protects against endotoxin (LPS)-induced lethality, hypoxia-reperfusion injury after liver transplantation, and D-galactosamine-mediated liver injury (1–4). Because depleting neutrophils or blocking their function before chemical or hypoxic challenge protects tissues, it has been concluded that neutrophils play a key role in pathophysiology (5–8). Neutrophils participate in injury by adhering and invading tissue and releasing superoxide, arachidonic acid metabolites, and proteases by processes that are largely calcium dependent (9–12).

Recently, it was shown that glycine inhibited LPS-mediated increases in $[Ca^{2+}]_i$ in Kupffer cells, the resident hepatic macrophage, by a mechanism dependent on extracellular chloride (13). This effect was reversed by strychnine, which is a well-characterized antagonist of the glycine-gated chloride channel in the nervous system (14). Activation of the neuronal glycine-gated chloride channel causes an influx of chloride and hyperpolarizes the nerve cell membrane, making opening of voltage-gated calcium channels on the cell surface more difficult (15, 16), thereby diminishing responses to a variety of agonists that depolarize the cell membrane. In an analogous fashion, glycine hyperpolarizes Kupffer cell membranes making calcium channels on the plasma membrane more difficult to open (17), which inhibits the production of cytokines (13). Glycine also causes an influx of radiolabeled chloride into the Kupffer cell (18). Moreover,

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molecular evidence (i.e., mRNA and protein) for the β-subunit of the glycine-gated chloride channel similar to the spinal cord glycine-gated chloride channel has been demonstrated in these macrophages (19). Taken together, these studies led to the conclusion that Kupffer cells contain glycine-gated chloride channels like the neuron. Because the Kupffer cell is a specialized leukocyte derived from the same pluripotent stem cell as neutrophils, the purpose of this study was to determine if neutrophils, which are very important in many inflammatory disease processes, contain a glycine-gated chloride channel. Preliminary accounts of this study have appeared elsewhere (20).

MATERIALS AND METHODS

Isolation and culture of neutrophils

Glycogen (1%) was dissolved in 35 ml of sterile saline and administered i.p. to female Sprague-Dawley rats (350–400 g) under light anesthesia with methoxyflourane. Four hours later, rats were reanesthetized, exsanguinated, and cells in the peritoneum were removed by lavage with 35 ml of sterile PBS containing 1000 U/L heparin. The suspension was centrifuged at 500*g* for 7 min, and erythrocytes were destroyed by hypotonic lysis in 0.15 M NH_4Cl for 2 min. Cells were resuspended and centrifuged again. The cell pellet was suspended in RPMI 1640 without glycine containing L-glutamine (1 mM), 10% heat-inactivated fetal bovine serum, penicillin (10 IU/ml), and streptomycin (10 μg/ml). Preparations were 95% viable and 98% pure as determined by trypan blue exclusion and Wright-Giemsa staining, respectively.

Measurement of intracellular calcium ($[Ca^{++}]_i$) in adherent neutrophils

Cells (5×10^5 cells/plate) were plated on glass coverslips and allowed to adhere for 20 min before replacing the media with fresh RPMI 1640 containing 10% FCS and antibiotics. Adherent neutrophils were incubated in modified Hank's balanced salt solution (m-HBSS, 110 mM NaCl, 5 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.6 mM glucose, 0.8 mM $MgSO_4{\cdot}7H_2O$, 1.26 mM $CaCl_2$, 4 mM $NaHCO_3$, 15 mM HEPES, pH 7.4) containing 5 mM Fura-2 acetooxymethyl ester for 60 min at room temperature. Coverslips were rinsed with m-HBSS and fresh buffer was added. Intracellular calcium was assessed by monitoring changes in fluorescence intensity of Fura-2 at excitation wavelengths of 340 and 380 nm with emission at 540 nm in individual cells. Values were corrected by subtracting system dark noise and autofluorescence by adding Mn^{++}. $[Ca^{2+}]_i$ was determined by the method of Grynkiewicz et al. (21) from the following equation:

$$[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](F_o/F_s)$$

where F_o/F_s is the ratio of fluorescence intensities evoked by 380 nm light from Fura-2 pentapotassium salt in a buffered salt solution containing nanomolar Ca^{2+} ($[Ca^{2+}]_{min}$) or millimolar calcium ($[Ca^{2+}]_{max}$). *R* is the ratio of fluorescence intensities at 340 and 380 nm. R_{max} and R_{min} are the values of *R* at $[Ca^{2+}]_{max}$ and $[Ca^{2+}]_{min}$, respectively. The dissociation constant (K_d) used was 135 nM (21). Peak increases in calcium concentration were consistently achieved within 30 s after agonist addition.

Measurement of ^{36}chloride uptake by neutrophils

Assays for uptake of ^{36}Cl used an adaptation of a method for neurons described by Schwartz et al. (22) modified by Morrow and Paul (23). In brief, neutrophils (2×10^6 cells/ml) were plated on glass coverslips and allowed to adhere for 1 h at 37°C. Media was replaced with buffer (20 mM HEPES, 118 mM NaCl, 4.7 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 10 mM glucose) and allowed to equilibrate for 10 min at room temperature. Coverslips were gently blotted dry and incubated in a petri dish with 2 ml of buffer containing 2 μCi/ml ^{36}Cl in the presence of glycine (0–2 mM) and/or strychnine (1 μM or 1 mM) for 5 s. Chloride influx was linear between 2–10 s; thus, a 5 s incubation time was chosen for all experiments. Strychnine was dissolved in DMSO, and the final concentrations of DMSO (0.002%) used in these studies had no effect on ^{36}Cl movement. Chloride flux was terminated by washing the coverslip with ice-cold buffer for 3 s followed by a second wash for 7 s (23). Coverslips were placed in scintillation vials and protein was solubilized by adding 1.6 ml of NaOH (0.2 M) for 2 h. An aliquot (0.16 ml) was taken for determination of protein by the method of Lowry (24). Ecolume (10 ml) was added and radioactivity was determined by scintillation spectroscopy. Flux measured in glycine-free buffer was subtracted from all values to account for basal chloride movement across the cell membrane as well as trapped radioactive chloride.

Measurement of superoxide release

Superoxide production was measured from the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* as described previously (25). Incubation volume was 1.0 ml, and the concentration of neutrophils was 2.0×10^6 cells/ml. Initially, cells were incubated with glycine (1 mM) for 5 min at 24°C. Cytochalasin B (4 μM, final concentration) was added to each tube and incubation continued at 37°C for 5 min to disrupt actin filaments. Formyl-methionine-leucine–phenylalanine (FMLP) (1 μM) was added and the incubation continued at 37°C for 30 min. The amount of reduced ferricytochrome *c* was determined spectrophotometrically at 550 nm. The difference in absorption between the samples incubated in the presence and absence of SOD (85 U/ml) was used to determine the amount of superoxide produced, using an extinction coefficient for reduced ferricytochrome *c* of $18500{\cdot}cm^{-1}{\cdot}M^{-1}$ (26).

RESULTS

Effects of FMLP and LPS on $[Ca^{2+}]_i$ in adherent neutrophils

Intracellular calcium in individual neutrophils was measured fluorometrically using the calcium indicator Fura-2. Representative traces depicted in **Fig. 1** show transient increases in $[Ca^{2+}]_i$ after treatment with FMLP (1 μM, Fig. 1*A*) or LPS (100 μg/ml +5% rat serum, Fig. 1*B*). The effect of LPS was dependent on rat serum because there is an absolute requirement for a species-specific LPS binding protein for binding to CD-14 to occur (27). The addition of rat serum alone had no effect on $[Ca^{2+}]_i$ in neutrophils (data not shown). After exposure to either agonist, basal values near 30 nM $[Ca^{2+}]_i$ increased rapidly

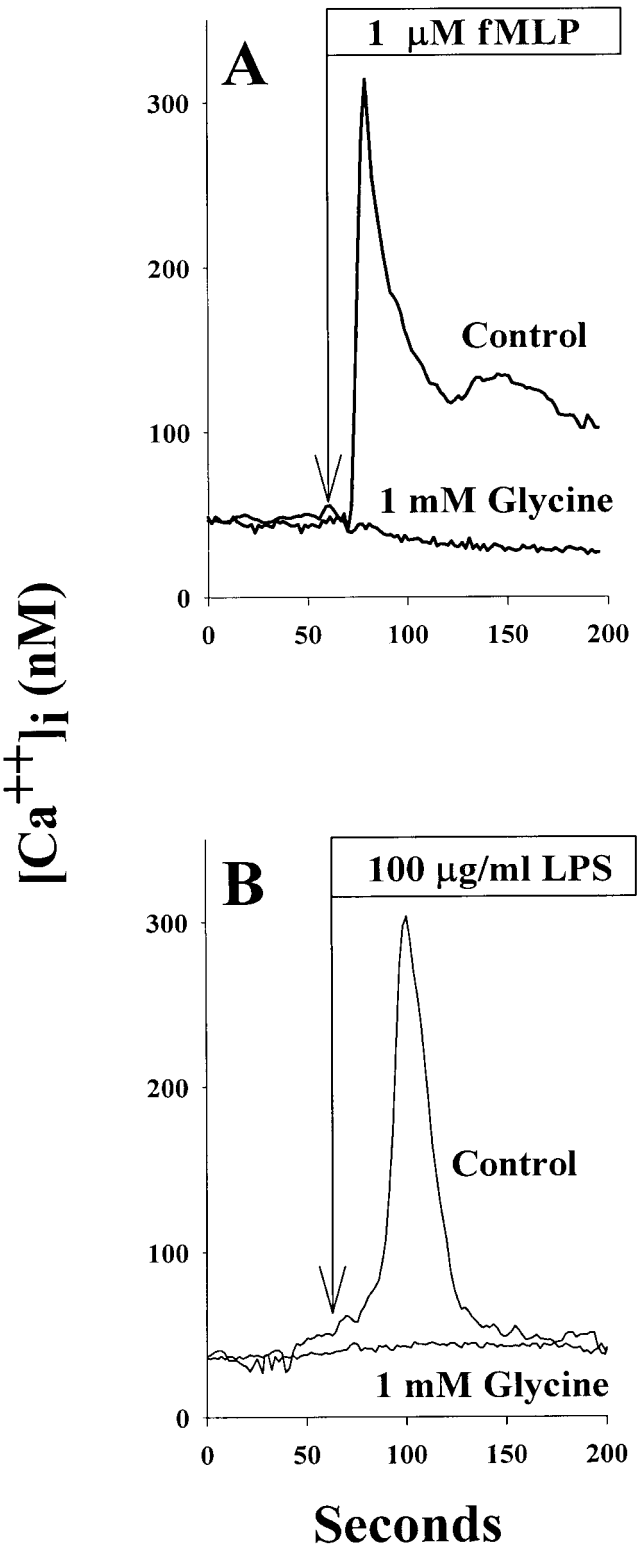

Figure 1. The effect of glycine on FMLP- and LPS-induced increases in $[Ca^{++}]_i$ in neutrophils. Neutrophils were isolated and cultured as described in Materials and Methods. After loading with Fura-2 as described in Materials and Methods, cells were incubated for 3 min in modified-HBSS in the presence or absence of glycine (1 mM). The buffer was removed and a solution containing the same concentration of glycine and (*A*) 1 μM FMLP or (*B*) 100 μg/ml LPS was added. Data shown are representative experiments repeated 4–6 times.

and reached peak values within 30 s. FMLP increased $[Ca^{2+}]_i$ to maximal values of ~275 nM followed by a decline that reached a plateau at ~175 nM within 1 min (Fig. 1*A*). On the other hand, LPS (Fig. 1*B*) caused a transient increase in $[Ca^{2+}]_I$, which peaked at 250 nM and returned to basal values within 1 min. Moreover, incubation with 1 mM glycine (see below) for 3 min had no effect on basal calcium levels but totally prevented agonist-induced increases in $[Ca^{2+}]_i$ in neutrophils (Fig. 1).

Concentration-response curves for agonists on increases in $[Ca^{2+}]_i$

The effect of various concentrations of FMLP (**Fig. 2*A***) and LPS (Fig. 2*B*) on $[Ca^{2+}]_i$ in single adherent neutrophils were determined in experiments typified by Fig. 1. The effect of FMLP was maximal at 1 μM with an ED_{50} of 0.44 μM. LPS increased $[Ca^{2+}]_i$ maximally at 100 μg/ml and had an ED_{50} of 45 μg/ml.

The effect of glycine on agonist-induced increases in $[Ca^{2+}]_i$

Neutrophils were incubated in m-HBSS containing glycine (0–2 mM) for 3 min before and during addition of agonist (**Fig. 3**). Glycine blunted the increase in $[Ca^{2+}]_i$ in neutrophils stimulated with either FMLP (1 μM, Fig. 3*A*) or LPS (100 μg/ml, Fig. 3*B*) and was not toxic to cells at any concentration tested as determined by trypan blue exclusion. This prevention of agonist-induced increases in $[Ca^{2+}]_i$ was linear between 0 an 1 mM glycine treatment. From linear regression analysis, the IC_{50} values for blunting increases in $[Ca^{2+}]_i$ after addition the FMLP or LPS were both ~0.3 mM glycine.

It appeared that glycine prevented the agonist-induced increases in $[Ca^{2+}]_i$ almost completely, suggesting that glycine blunted the release of calcium from intracellular stores as well as calcium influx from extracellular space Therefore, the effect of glycine on the calcium increase from intracellular stores was evaluated in the absence of extracellular calcium. Removal of the extracellular calcium and addition of EGTA had little effect on the peak $[Ca^{2+}]_i$ after LPS stimulation compared with cells stimulated in the presence of calcium (**Fig. 4**). However, the agonist-induced increase in $[Ca^{2+}]_i$ was prevented completely by the addition of 1 mM glycine in both the presence or absence of calcium.

The role of extracellular chloride in glycine-mediated inhibition of agonist-induced increases in $[Ca^{2+}]_i$

It is known that glycine activates a glycine-gated chloride channel in neurons and Kupffer cells,

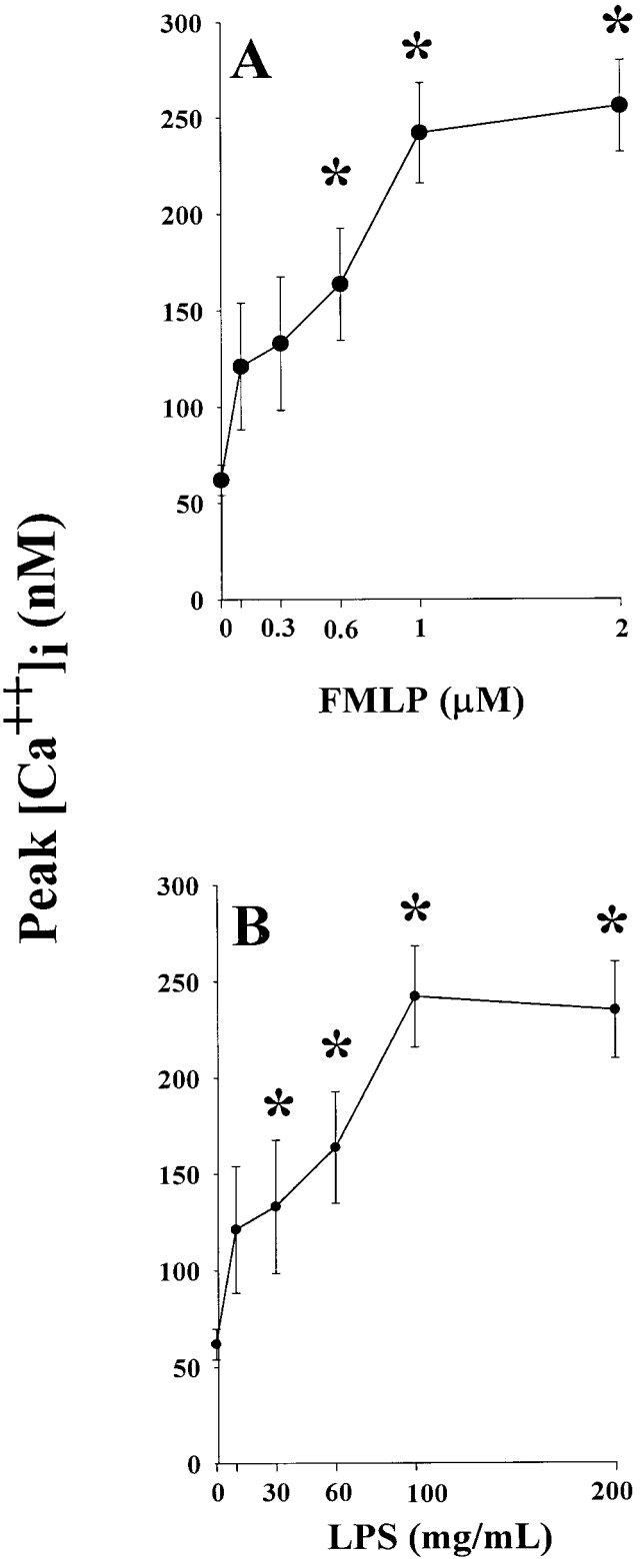

Figure 2. Effect of FMLP or LPS on peak $[Ca^{++}]_i$ concentrations in neutrophils. Cells were isolated, cultured, and loaded with Fura-2 as described in Materials and Methods. Basal $[Ca^{2+}]_i$ was obtained during incubation in m-HBSS. The buffer was replaced with m-HBSS containing (*A*) FMLP (0–3 μM) or (*B*) LPS (0–300 μg/ml), and the peak $[Ca^{2+}]_i$ concentration was determined as the maximal calcium concentration achieved within 30 s following stimulation as described in Materials and Methods. Data are means ± SE (n=4–6). *Significantly different from peak intracellular calcium in cells that were not treated with agonist ($P<0.05$) by one-way analysis of variance (ANOVA) and Bonferroni's *post hoc* test.

leading to chloride influx that attenuates increases in $[Ca^{2+}]_i$ (13, 15, 16). It was hypothesized that glycine prevents FMLP- and LPS-mediated increases in $[Ca^{2+}]_i$ in the neutrophil by a similar mechanism. To test this hypothesis, chloride in the buffer was substituted with gluconate. Indeed, in chloride-free buffer, glycine (1 mM) was unable to block agonist-induced increases in $[Ca^{2+}]_i$ when cells were stimulated either with FMLP or LPS (**Fig. 5**).

Strychnine, at micromolar concentrations, is an inhibitor of the glycine-gated chloride channel in the neuron and Kupffer cell (13, 14). Strychnine (1 μM) was added to cells before stimulation with FMLP or LPS to test the hypothesis that it would also reverse the effect of glycine in neutrophils (**Fig. 6**). As shown above, both FMLP and LPS caused an increase in $[Ca^{2+}]_i$ that was totally prevented by glycine. Indeed, the addition of 1 μM strychnine reversed the effect of glycine and restored agonist-induced increases in $[Ca^{2+}]_i$ to near normal levels.

In other cells, such as isolated kidney tubules and Kupffer cells, strychnine inhibits the effect of glycine at low concentrations (micromolar range) but has agonist actions like glycine in the millimolar range (13, 28). Indeed, high concentrations of strychnine (1 mM) prevented agonist-induced increases in $[Ca^{2+}]_i$ like glycine with both FMLP (80±25 nM) or LPS (61±16 nM) (Fig. 6).

Radiolabeled chloride is used routinely in cells to provide hard evidence for movement of ions from the extracellular to the intracellular space (22, 23). Accordingly, adherent neutrophils were incubated with ^{36}Cl and increasing concentrations of glycine (**Fig. 7*A***). Glycine caused a dose-dependent increase in ^{36}Cl influx. The ED_{50} value for glycine-stimulated chloride influx was 0.23 mM, which is essentially the same value as the IC_{50} for inhibition of agonist-induced increases in $[Ca^{2+}]_i$ by glycine. Furthermore, the addition of 1 μM strychnine prevented chloride influx due to glycine almost completely, and high concentrations of strychnine (1 mM) activated chloride influx essentially like glycine (Fig. 7*B*) .

Effect of glycine on superoxide production by neutrophils

It is known that production of superoxide by neutrophils is, in part, calcium dependent (29). To determine if glycine could inhibit calcium-dependent functions of neutrophils, the effect of glycine on

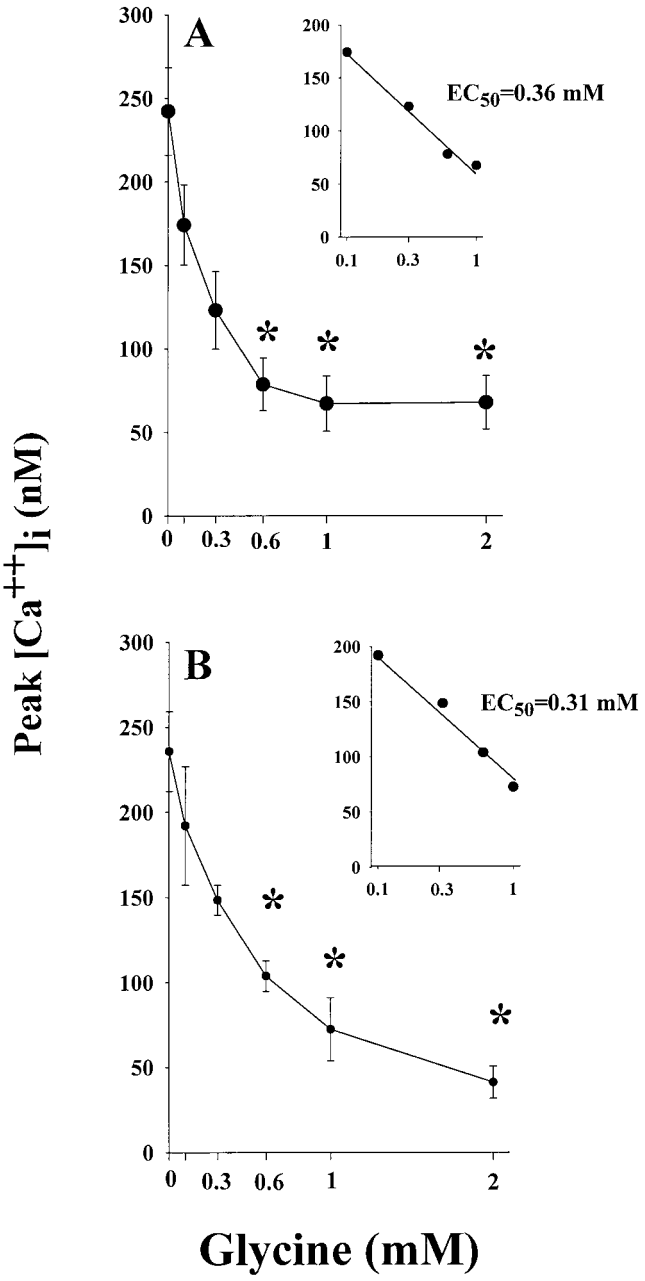

Figure 3. The effect of glycine on FMLP- and LPS-induced increases in $[Ca^{++}]_i$ in neutrophils. Neutrophils were isolated, cultured, and loaded with Fura-2 as described in Materials and Methods. Cells were incubated for 3 min in m-HBSS containing glycine (0–2 mM). The buffer was then replaced with m-HBSS with (*A*) FMLP (1 μM) or (*B*) LPS (100 μg/ml) containing the same concentration of glycine. Peak intracellular calcium concentration was determined as described in Materials and Methods. Insets reflect intracellular calcium concentrations as the logarithmic function of glycine concentration and are shown to demonstrate linearity. Linear regression was performed to calculate glycine EC_{50} values. Data are means ± SE (n=4–6). *Significantly different from cells incubated without glycine (P<0.05) by one-way ANOVA for each concentration and Bonferroni's *post hoc* test.

superoxide production was studied. FMLP stimulated neutrophils to produce nearly 7.6 ± 0.4 nmol superoxide/10^6 cells/30 min. Glycine (1 mM) blunted superoxide production in isolated neutrophils significantly to 4.8 ± 0.5 nmol superoxide/10^6 cells/30 min (n=4–6 per group, P<0.05, Student's t test).

DISCUSSION

Endotoxin increases calcium in neutrophils

FMLP and LPS caused dose-dependent increases in peak $[Ca^{2+}]_i$ in neutrophils with slightly different kinetics (Figs. 1 and 2). FMLP caused a rapid increase in $[Ca^{2+}]_i$ within seconds followed by a gradual decrease, which reached a plateau and never returned to basal values, confirming work by others (30). The initial increase in calcium most likely arises from intracellular stores, whereas the sustained in-

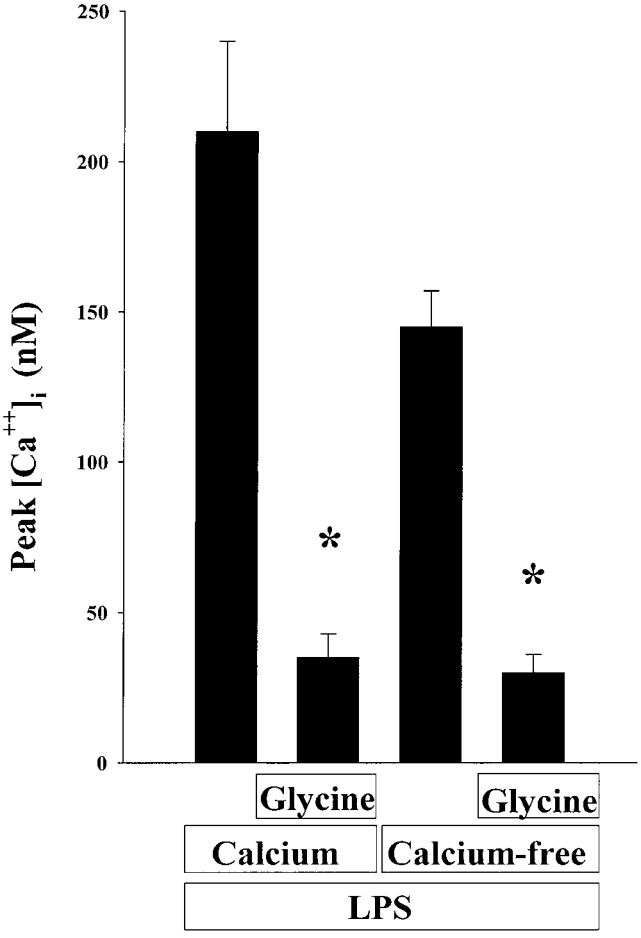

Figure 4. The lack of dependence of agonist-induced increases in $[Ca^{++}]_i$ by glycine on extracellular calcium. Neutrophils were isolated, cultured, and loaded with Fura-2 as described in Materials and Methods. Cells were incubated in m-HBSS with or without calcium or containing glycine (1 mM) for 3 min. Buffer was replaced with the same buffer containing LPS (100 μg/ml) and peak intracellular calcium concentrations were determined as described above in Materials and Methods. Data are means ± SE (n=8). *Significantly different from basal (P<0.05) by two-way ANOVA and Bonferroni's *post hoc* test.

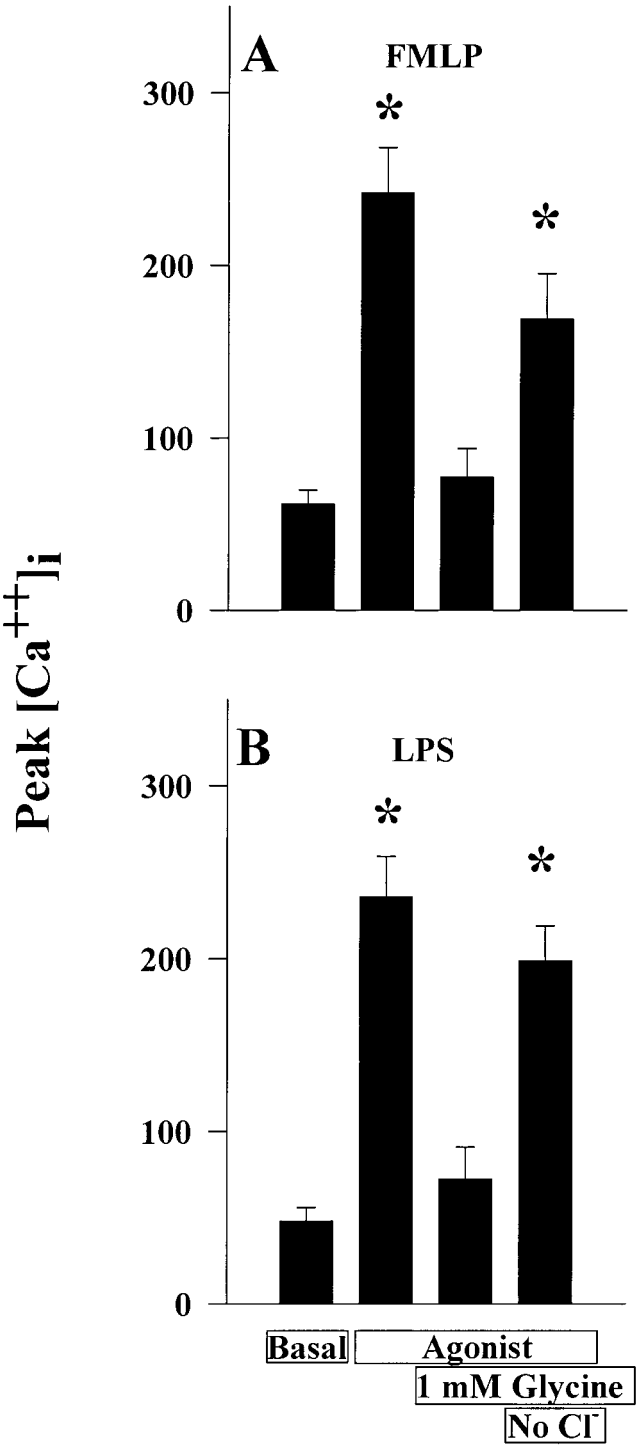

Figure 5. The effect of chloride-free buffer on the effect of glycine on $[Ca^{++}]_i$. Neutrophils were isolated, cultured, and loaded with Fura-2 as described in Materials and Methods. *A)* Cells were incubated in chloride-free buffer by substitution of sodium chloride with sodium gluconate for 3 min before the addition of glycine (1 mM). After incubation in chloride-free buffer containing glycine for 3 min, LPS (100 μg/mL) or FMLP (1 μM) was added and peak intracellular calcium concentrations were determined. Data are means ± SE (n=4–6). *Significantly different from basal (P<0.05) by two-way ANOVA and Bonferroni's *post hoc* test.

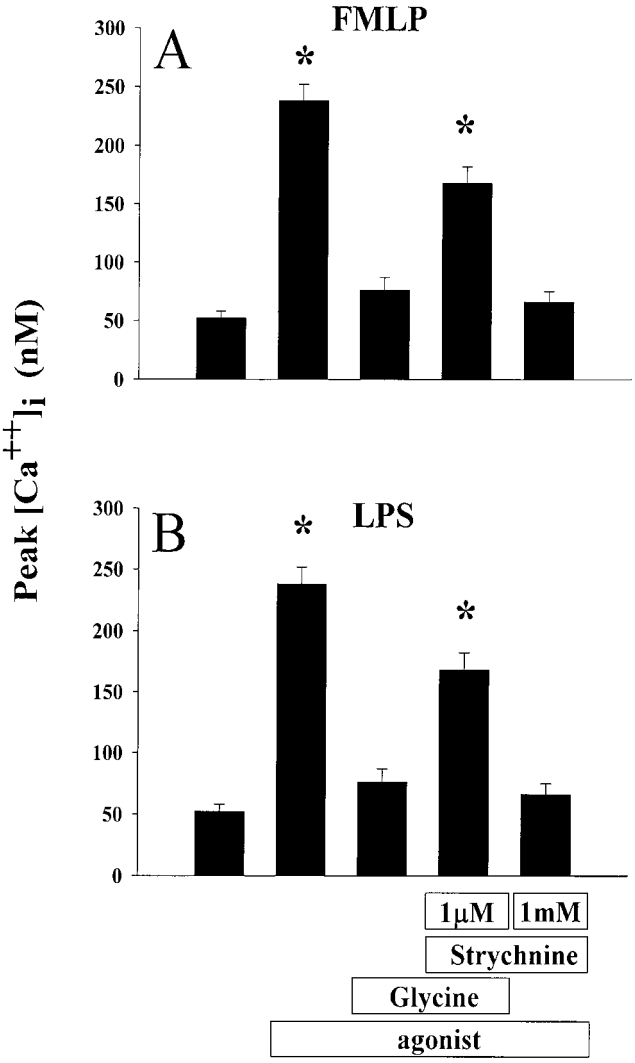

Figure 6. The effect of strychnine on glycine inhibition of increases in $[Ca^{++}]_i$. Cells were incubated in either low-dose strychnine (1 μM) before the addition of glycine (1 mM) or high-dose (1 mM) strychnine for 3 min. After incubation in strychine buffer containing glycine for 3 min, (*A*) FMLP (1 μM) or (*B*) LPS (100 μg/mL) was added and peak intracellular calcium concentrations were determined fluorometrically. Results are from a typical experiment repeated 4 times. The effect of agonist alone ± SE is shown for comparison (peak $[Ca^{2+}]_i$ in control). *Significantly different from control (P<0.05) by one-way ANOVA and Bonferroni's *post hoc* test.

crease is the result of influx of calcium from the extracellular space (31). Although LPS also caused an increase in $[Ca^{2+}]_i$, the mechanism of LPS signaling and calcium mobilization in neutrophils is not clearly understood. This is the first study showing that LPS can increase calcium in neutrophils; however, relatively high concentrations of LPS (100 μg/ml) are needed. LPS concentrations commonly used in *in vitro* studies (32) are higher than physiological circulating LPS concentrations. Thus, the physiological significance of the activation of neutrophils by LPS is not clear but may be important in

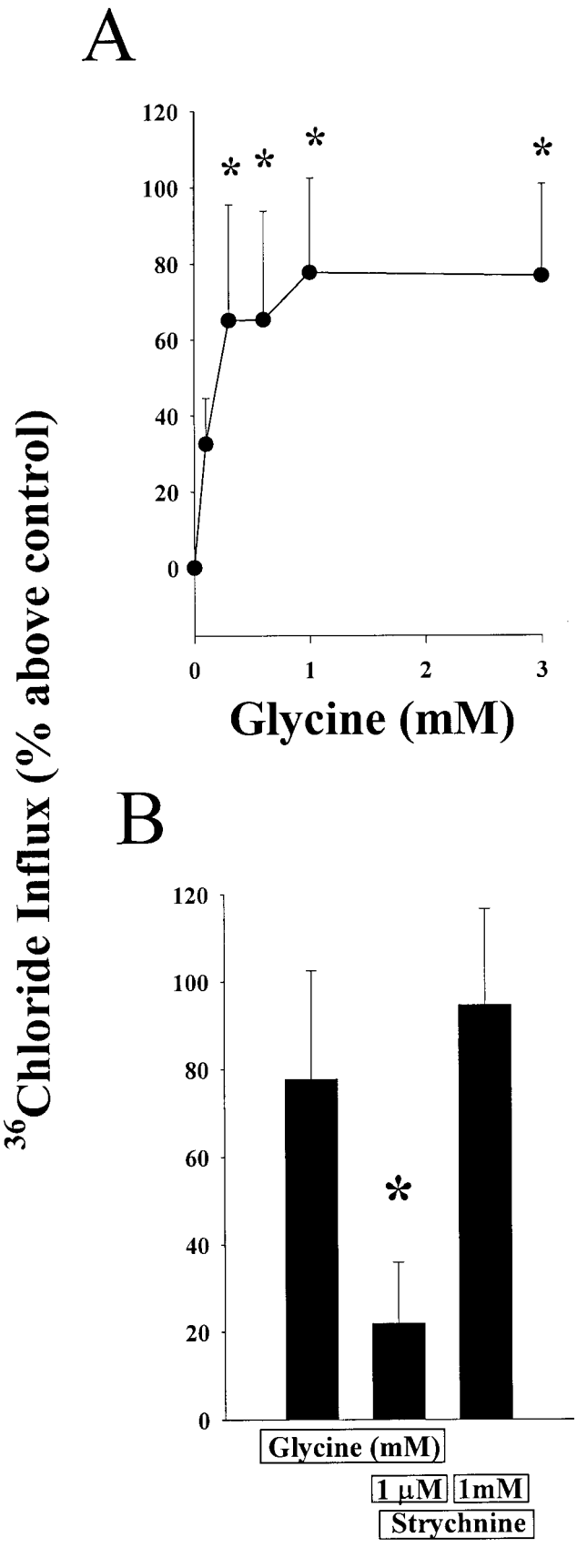

Figure 7. The effect of glycine and strychnine on radiolabeled chloride influx in neutrophils. Cultured neutrophils were incubated with ^{36}Cl (2 μCi/ml) in the presence or absence of (*A*) glycine (0–2 mM) or (*B*) strychnine (1 μM or 1 mM). Data are presented as a percentage of $^{36}Cl^-$ movement in the absence of glycine (no additions) of eight independent experiments and are means ± SE. *Significantly different from control ($P<0.05$) by one-way ANOVA and Bonferroni's *post hoc* test.

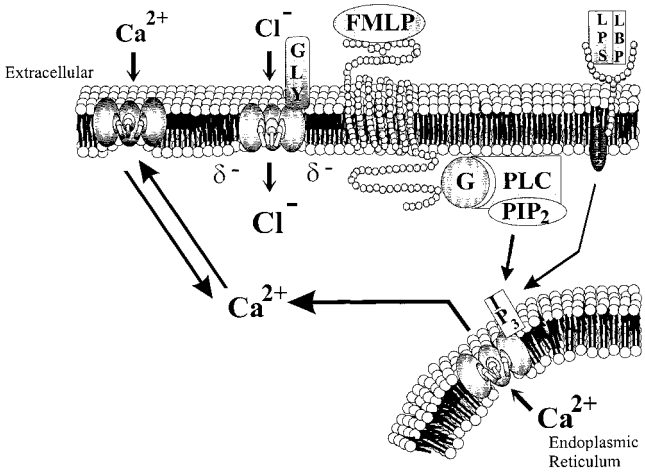

Figure 8. Proposed mechanism for inhibition of agonist-induced increases in $[Ca^{++}]_i$ by glycine. Glycine activates a chloride channel in the plasma membrane of neutrophils, which leads to influx of chloride ions that hyperpolarizes the cell membrane. It is hypothesized that changes in membrane potential across both the cell and endoplasmic reticulum membranes decrease the open probability of calcium channels, thereby blocking movement of calcium across the plasma membrane and release from intracellular calcium stores after addition of FMLP or LPS. Furthermore, activation of signal transduction pathways in the cell that involve increases in $[Ca^{2+}]_i$ would be blunted, resulting in an inhibition of neutrophil function.

disease states such as septic shock where LPS levels are elevated (33).

Neutrophils contain a glycine-gated chloride channel

The primary goal of this study was to determine if neutrophils contain a glycine-gated chloride channel analogous to the channel in the neuron and Kupffer cell (4, 34). It was hypothesized that glycine would prevent increases in $[Ca^{2+}]_i$ by hyperpolarizing the cell membrane, thereby preventing activation of voltage-dependent calcium channels. Indeed, glycine blunted FMLP- and LPS-stimulated increases in $[Ca^{2+}]_i$ with an IC_{50} ~0.3 mM (Fig. 3). This value is similar to the IC_{50} values for prevention of LPS toxicity and inhibition of activation of Kupffer cells by glycine and only about twice as high as normal circulating concentrations. These blood levels are easily achieved with a diet enriched with glycine (1). In addition, it is concluded that glycine not only affects influx of calcium from the extracellular space but also prevents release from intracellular stores,

because glycine totally prevented increases in $[Ca^{2+}]_i$ due to LPS and FMLP both in the presence and absence of extracellular calcium (Fig. 4). The mechanism for this effect of glycine remains unclear. It has been suggested that the IP_3-gated chloride channel on the endoplasmic reticulum may be inactivated when the potential difference across the membrane is increased (35). Thus, it is possible that influx of chloride across the cell membrane also increases the potential difference across the endoplasmic reticulum making the IP_3-receptor more difficult to open (see **Fig. 8**).

Strychnine is a well known antagonist of glycine-gated chloride channels (13, 14). Here, 1 μM strychnine reversed the effect of glycine, restoring the increase in $[Ca^{2+}]_i$ to near control values (Fig. 6). These data provide pharmacological evidence for the presence of glycine-gated chloride channels in neutrophils. High concentrations of strychnine (1 mM) have been shown to be protective against hypoxic injury in the perfused liver and isolated renal proximal tubules as well as blunt increases in $[Ca^{2+}]_i$ in Kupffer cells, which is apparently paradoxical to its effects on the glycine-gated chloride channel. (13, 28, 36). In this study, a high concentration of strychnine also prevented increases in $[Ca^{2+}]_i$ due to LPS and FMLP and caused influx of chloride, essentially like glycine. Based on these data, it is concluded that strychnine at high concentrations is also an agonist for the glycine-gated chloride channel in the neutrophil.

If the effect of glycine is a result of activation of a glycine-gated chloride channel in the neutrophil, it would be dependent on the presence of extracellular chloride. Indeed, substitution of chloride with an impermeable anion gluconate prevented the inhibitory effect of glycine on agonist-induced increases in $[Ca^{2+}]_i$ almost completely (Fig. 5). Moreover, glycine increased radiolabeled chloride movement into the neutrophil in a dose-dependent manner (Fig. 7) with an ED_{50} value almost identical to the IC_{50} value for prevention of LPS- and FMLP-induced increases in $[Ca^{2+}]_i$. Strychnine (1 μM) prevented the influx of chloride, as expected, providing more evidence that glycine blunts increases in $[Ca^{2+}]_i$ by activating a glycine-gated chloride channel. Thus, it is concluded that glycine activates a chloride channel in the neutrophil.

Based on the data presented above, it is hypothesized that glycine inhibits processes in the neutrophil that are dependent on increases in $[Ca^{2+}]_i$, such as activation of NADPH oxidase and the production of superoxide radicals. Indeed, glycine blunted superoxide production by neutrophils stimulated with FMLP. The fact that glycine did not completely prevent superoxide release from the neutrophil is not surprising, because there are calcium-independent pathways (e.g., activation of tyrosine kinases) that also mediate superoxide release from the neutrophil. However, increases in intracellular calcium are required for optimal superoxide generation from FMLP-stimulated neutrophils (9–12, 29).

Proposed mechanism of action of glycine

Based on the data presented here, the following mechanism of action of glycine in neutrophils is proposed (Fig. 8). When cells are stimulated with either LPS or FMLP, signal transduction pathways are activated that change the potential differences across the cell membrane. In the presence of glycine, a glycine-gated chloride channel is activated causing an influx of chloride, leading to hyperpolarization of the neutrophil membrane. The increase in membrane potential caused by agonists is blunted by glycine, which decreases the opening time of voltage-dependent calcium channels on the cell membrane and inhibits the influx of calcium. In addition, influx of chloride could also inactivate the IP_3-gated calcium channel and blunt release of calcium from intracellular stores. Decreased calcium influx blunts the activation of calcium-dependent pathways in the cell inhibiting the function of the phagocyte.

Clinical applications of glycine

Glycine has several benefits that make its clinical application appealing. First, glycine can be administered in the diet without side effects (37). Second, data from this and previous studies with Kupffer cells and alveolar macrophages (13, 38) show that glycine is antiinflammatory and could be useful in many disease states which are dependent on the activation of neutrophils and macrophages (4). Indeed, it has been shown previously that glycine prevents lethality due to LPS and in a model of ischemia-reperfusion injury to the liver *in vivo* followed by administration of LPS (two-hit model) (1). This model mimics the increase in sensitivity of trauma patients to LPS. Moreover, glycine was shown to be protective in the classical galactosamine toxicity model, presumably by inactivation of Kupffer cells (3). Currently, glycine has only been shown to be effective prophylacticly; however, it could be potentially useful in a wide variety of inflammatory processes where neutrophil infiltration contributes to toxicity. FJ

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REFERENCES

1. Ikejima, K., Iimuro, Y., Forman, D. T., and Thurman, R. G. (1996) A diet containing glycine improves survival in endotoxin shock in the rat. *Am. J. Physiol.* **271,** G97–G103
2. den Butter, G., Lindell, S. L., Sumimoto, R., Schilling, M. K., Southard, J. H., and Belzer, F. O. (1993) Effect of glycine in dog and rat liver transplantation. *Transplantation* **56,** 817–822
3. Stachlewitz, R. F., Seabra, V., Bradford, B. U., Bradham, C. A., Rusyn, I., Germolec, D., and Thurman, R. G. (1999) Glycine and uridine prevent D-galactosamine hepatotoxicity in the rat: role of Kupffer cells. *Hepatology* **29,** 737–745
4. Wheeler, M. D., Ikejima, K., Enomoto, N., Stachlewitz, R. F., Seabra, V., Zhong, Z., Schemmer, P., Rose, M. L., Rusyn, I., Bradford, B. U., and Thurman, R. G. (1999) Glycine: a new anti-inflammatory immunonutrient. *Cell. Mol. Life Sci.* In press
5. Malech, H. J., and Gallin, J. I. (1987) Current concepts: immunology. Neutrophils in human diseases. *N. Engl. J. Med.* **317,** 687–694
6. Jaeschke, H., Farhood, A., and Smith, C. W. (1990) Neutrophils contribute to ischemia/reperfusion injury in rat liver *in vivo*. *FASEB J.* **4,** 3355–3359
7. Lemasters, J. J., and Thurman, R. G. (1991) Reperfusion injury: a role for neutrophils. *Hepatology Elsewhere* **14,** 952–955
8. Hewett, J. A., Schultze, A. E., VanCise, S., and Roth, R. A. (1992) Neutrophil depletion protects against liver injury from bacterial endotoxin. *Lab. Invest.* **66,** 347–361
9. Varani, J., Ginsburg, I., Schuger, L., Gibbs, D. F., Bromberg, J., Johnson, K. J., Ryan, U. S., and Ward, P. A. (1989) Endothelial cell killing by neutrophils: synergistic interaction of oxygen products and proteases. *Am. J. Pathol.* **135,** 435–438
10. Thelen, M., Dewald, B., and Baggiolini, M. (1993) Neutrophil signal transduction and activation of the respiratory burst. *Physiol. Rev.* **73,** 797–821
11. Sklar, L. A., and Oades, Z. G. (1985) Signal transduction and ligand-receptor dynamics in the neutrophil. *J. Biol. Chem.* **260,** 11468–11475
12. O'Flaherty, J. T., Rossi, A. G., Jacobson, D. P., and Redman, J. F. (1991) Roles of Ca^{2+} in human neutrophil responses to receptor agonists. *Biochem. J.* **278,** 705–711
13. Ikejima, K., Qu, W., Stachlewitz, R. F., and Thurman, R. G. (1997) Kupffer cells contain a glycine-gated chloride channel. *Am. J. Physiol.* **272,** G1581–G1586
14. Ito, S., and Cherubini, E. (1991) Strychnine-sensitive glycine responses of neonatal rat hippocampal neurons. *J. Physiol.* **440,** 67–83
15. Langosch, D., Becker, C.-M., and Betz, H. (1990) The inhibitory glycine receptor: a ligand-gated chloride channel of the central nervous system. *Eur. J. Biochem.* **194,** 1–8
16. Betz, H., Langosch, D., Hoch, W., Prior, P., Pribilla, I., Kuhse, J., Schmieden, V., Malosio, M.-L., Matzenbach, B., Holzinger, F., Kuryatov, A., Schmitt, B., Maulet, Y., and Becker, C.-M. (1991) Structure and expression of inhibitory glycine receptors. In *Neuroreceptor Mechanisms in Brain* (Kito, S., ed) pp. 421–429, Plenum, New York
17. Hijioka, T., Rosenberg, R. L., Lemasters, J. J., and Thurman, R. G. (1992) Kupffer cells contain voltage-dependent calcium channels. *Mol. Pharmacol.* **41,** 435–440
18. Yin, M., Ikejima, K., Arteel, G. E., Seabra, V., Bradford, B. U., Kono, H., Roussyn, I., and Thurman, R. G. (1998) Glycine accelerates recovery from alcohol-induced liver injury. *J. Pharmacol. Exp. Ther.* **286,** 1014–1019
19. Wheeler, M. D., Seabra, V., and Thurman, R. G. (1999) Molecular evidence for glycine-gated chloride channel in Kupffer cells. In *Cells of the Hepatic Sinusoid* (Wisse, E., Knook, D. L., and Wake, K., eds) The Kupffer Cell Foundation, Leiden, The Netherlands. In press
20. Stachlewitz, R. F., Ikejima, K., and Thurman, R. G. (1995) Increases in intracellular calcium in neutrophils (PMNs) due to formyl-methionine-leucine-phenylalanine (FMLP) and endotoxin are blocked completely by glycine. *Hepatology* **22,** 1105
21. Gyrnkiewicz, G., Poenie, M., and Tsien, R. W. (1985) A new generation of calcium indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260,** 3440–3450
22. Schwartz, R. D., Paul, S. M., and Majewska, M. D. (1986) Factors modulating the sensitivity of a GABA-gated chloride channel. *Clin. Neuropharmacol.* **9,** 389–391
23. Morrow, A. L., and Paul, S. M. (1988) Benzodiazepin enhancement of gamma-aminobutyric acid mediated Cl^- ion flux in rat brain synaptoneurosomes. *J. Neurochem.* **50,** 302–306
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275
25. Babior, B. R., Knipes, R., and Curnette, J. (1973) Biological defense mechanisms: the production by leukocytes of superoxide, a potential bacterialcidal agent. *J. Clin. Invest.* **52,** 741–744
26. Margoliash, E., and Frohwirt, N. (1959) Spectrum of horse cytochrome C. *Biochem. J.* **71,** 570–572
27. Hailman, E., Vasselon, T., Kelley, M., Busse, L. A., Hu, M. C., Lichenstein, H. S., Detmers, P. A., and Wright, S. D. (1996) Stimulation of macrophages and neutrophils by complexes of lipopolysaccharide and soluble CD14. *J. Immunol.* **156,** 4384–4390
28. Miller, G. W., Lock, E. A., and Schnellmann, R. G. (1994) Strychnine and glycine protect renal proximal tubules from various nephrotoxicants and act in the late phase of necrotic cell injury. *Toxicol. Appl. Pharmacol.* **125,** 192–197
29. Irita, K., Fujita, I., Takeshige, K., Minakami, S., and Yoshitake, J. (1986) Calcium channel antagonist induced inhibition of superoxide production in human neutrophils. *Biochem. Pharmacol.* **35,** 3465–3471
30. Jaconi, E. E. M., Rivest, R. W., Schlegel, W., Wollheim, C. B., Pittet, D. P., and Lew, D. (1988) Spontaneous and chemoattractant-induced oscillations of cytosolic free calcium in single adherent human neutrophils. *J. Biol. Chem.* **263,** 10557–10560
31. Krause, K., Demaurex, N., Jaconi, M., and Lew, D. P. (1993) Ion channels and receptor-mediated Ca^{2+} influx in neutrophil granulocytes. *Blood Cells* **19,** 165–173
32. Van Deventer, S. J. H., Ten Cate, J. W., and Tytgat, G. N. J. (1988) Intestinal endotoxemia: clinical significance. *Gastroenterology* **94,** 825–831
33. Bode, C., Kugler, V., and Bode, J. C. (1987) Endotoxemia in patients with alcoholic and non-alcoholic cirrhosis and in subjects with no evidence of chronic liver disease following acute alcohol excess. *J. Hepatol.* **4,** 8–14
34. Rajendra, S., Lynch, J. W., and Schofield, P. R. (1997) The glycine receptor. *Pharmacol. Ther.* **73,** 121–146
35. Mak, D. D., and Foskett, J. K. (1997) Single-channel kinetics, inactivation, and spatial distribution of inositol trisphosphate(IP_3) receptors in xenopus oocyte nucleus. *J. Gen. Physiol.* **109,** 571–587
36. Zhong, Z., Jones, S., and Thurman, R. G. (1996) Glycine minimizes reperfusion injury in a low-flow, reflow liver perfusion model in the rat. *Am. J. Physiol.* **270,** G332–G338
37. Rosse, R. B., Theut, S. K., Banay-Schwartz, M., Leighton, M., Scarella, E., Cohen, C. G., and Deutsch, S. I. (1989) Glycine adjuvant therapy to conventional neuroleptic treatment in schizophrenia: an open-label, pilot study. *Clin. Neuropharmacol.* **12,** 416–424
38. Wheeler, M. D., and Thurman, R. G. (1999) Production of superoxide and TNFα from alveolar macrophages is blunted by glycine. *Am. J. Physiol.* **277,** L952–L959

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