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Fenspiride and Membrane Transduction Signals in Rat Alveolar Macrophages

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ABSTRACT. Fenspiride inhibits the calcium signal evoked by the inflammatory peptide formyl-Met-Leu-Phe (fMLP) in peritoneal macrophages, but at concentrations (≈1 mM) far above the therapeutic range (≈1 μM). Here, in rat alveolar macrophages, high fenspiride concentrations (1 mM) were required to inhibit the calcium signals evoked by the calcium agonist Bay K8644 or by ionomycin. Moreover, fenspiride (1 mM) was a poor inhibitor of the cell membrane depolarization induced by gramicidine D. By contrast, fenspiride blocked Na^+-H^+ antiport activation by (i) fMLP with an IC_{50} = 3.1 ± 1.9 nM and (ii) PMA (phorbol 12-myristate 13-acetate) with an IC_{50} = 9.2 ± 3.1 nM. Finally, protein kinase C (PKC) activity of macrophage homogenate was not significantly modified by 10 or 100 μM fenspiride (at 100 μM: 2.57 ± 1.60 vs. 2.80 ± 1.71 pmol/10^6 cells/min). In conclusion, fenspiride inhibits fMLP- and PMA-induced pH signals in rat alveolar macrophages, probably by acting distally on the PKC transduction signal. This pH antagonistic action may be relevant for the antiinflammatory mechanism of fenspiride and requires further investigation. BIOCHEM PHARMACOL **54**;2: 293–297, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. fenspiride; macrophage; Na^+-H^+ exchange; cytosolic calcium; membrane potential; protein kinase C

Fenspiride is an antiinflammatory agent used for the relief of upper and lower respiratory tract disease (for review see [1, 2]). Animal studies have shown that fenspiride is efficient in reducing acute carrageenin-induced pleuresy in the mouse [3] and zymosan-induced pleuresy in the rat [4]. Fenspiride was also reported to reduce the chronic bronchitis induced by SO_2 inhalation in rats [5].

At the molecular level, the mechanism of action of fenspiride is poorly understood. *In vitro* studies in guinea pig alveolar macrophages have demonstrated that fenspiride dose-dependently inhibits chemiluminescence and secretion of cyclo-oxygenase metabolites ([6], see also [1]). Pipy *et al.* [7] reported that fenspiride antagonizes the calcium signal induced by the chemotactic peptide formyl-Met-Leu-Phe (fMLP)† in peritoneal macrophages.

The role of alveolar macrophages in pulmonary pathophysiology has now been clearly established [6]. These cells play a key role in airway inflammation processes by releasing prostaglandins, thromboxane, oxygen free radicals, and platelet-activating factor (PAF). Therefore, by using rat alveolar macrophages here, we reexamined the action of fenspiride on the calcium signal evoked by fMLP and other agents. High fenspiride concentrations were required to inhibit such calcium signals. Thus, we investigated a potential action of fenspiride on other events linked to cell activation such as membrane depolarization and changes in cell pH.

MATERIALS AND METHODS

Rat Alveolar Macrophages

Male Wistar rats weighing 250–350 g (from CERJ, Le Genest St Isle, France) were anesthetized with sodium pentobarbital (100 mg/kg i.p.) and killed by bleeding (via a section of the aorta). The heart and lung block was excised, and the lungs were washed ten times via the trachea with 2 mL volumes of sterile pyrogen-free physiological saline. The obtained cell suspension was centrifuged for 7 min at 300 × *g*. The supernatant was discarded and the pellet resuspended. Cell suspensions were counted and diluted with Ringer solution to obtain 4.10^5 cells/mL. Ringer medium contained (mM): NaCl 145, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, MOPS(4-morpholinopropanesulfonic acid)-Tris 10 (pH = 7.4), glucose 5.

Measurement of pHi

Cytosolic pH (pH_i) was measured by using the fluorescent probe BCECF (2′,7′-bis(2-carboxyethyl)-5-(and-6)car-

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†*Abbreviations:* BCECF, 2′,7′-bis(2(carboxyethyl)-5-(and-6)carboxyfluorescein); BCECF-AM, BCECF-acetoxymethylester; $DiSBAC_2(3)$, bis-(1,3-diethylthiobarbituric acid) trimethine oxonol; fMLP, peptide formyl-Met-Leu-Phe; Fura 2, 1-[2-(5-carbaoxyoazol-2-yl)-6-aminobenzofuran-5-oxyl]2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N′,N′-tetraacetic acid; Fura 2-AM, Fura 2-acetoxymethylester; PAF, platelet-activating factor; pHi, cytosolic pH; PKC, protein kinase C; and PMA, phorbol 12-myristate 13-acetate.

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boxyfluorescein). Two hundred microliters of macrophage suspension were plated on 9 × 35 mm coverslips 1–3 hr before the experiments. Adherent macrophages were incubated for 15 min with Ringer medium containing 1 μmol/L of the membrane-permeant form BCECF-AM (BCECF-acetoxymethylester) at 37°C in the dark. After BCECF cell loading, the coverslips were washed three times by immersion in a Ringer solution and kept for 15 min at 37°C in the same Ringer medium to allow full BCECF-AM hydrolysis. The coverslips were mounted on a Teflon holder and placed at a ≈30° angle to the incident light beam in cuvettes containing 2 mL of Ringer solution. Fluorescence was recorded every 2 sec on a Shimadzu RF 5000 spectrofluorimeter (Roucaire, Vélizy-Villacoublay, France) at 508 nm (excitation wavelength) and 525 nm (emission wavelength). Background signal and macrophage autofluorescence were negligible (1% of the total fluorescence). pH calibration was performed in the presence of 10 μmol/L nigericin by using 135 mM KCl Ringer solutions of different pH.

Na^+:H^+ Exchange Activity

Cells were alkalinized by 15-min exposure to a Ringer solution where NaCl 25 mM was replaced by an equimolar amount of NH_4Cl and acidified by switching to ammonium-free, sodium-free Ringer solution (*N*-methyl-D-glucamine replaced Na). The initial rate of the subsequent recovery alkalinization was measured fluorimetrically by using the above protocol; the amiloride-sensitive fraction of this process was considered as a marker of Na^+:H^+ exchange activity.

Measurement of Membrane Potential

Membrane potential was fluorimetrically measured by using the fluorescent probe $DiSBAC_2(3)$ (bis-(1,3-diethylthiobarbituric acid)trimethine oxonol). Ten to 20 nM bis-oxonol were added to 10^6 cells/mL suspended in 2 mL Ringer solution. Fluorescence was measured in a Shimadzu RF 5000 spectrofluorimeter (excitation = 553 nm; emission = 580 nm), using slits at 5 nm. Calibration was done by addition of gramicidine D at a final concentration of 1 μg/mL.

Measurement of Cytosolic Calcium

Cytosolic free calcium concentration was monitored by using the calcium-sensitive dye Fura 2 (1-[2-(5-carbaoxyoazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid). Coverslip-adherent macrophages were loaded with 4 μM Fura 2-AM in Ringer solution for 30 min at 37°C in the dark. Cells were washed three times with Ringer medium and transferred to dye-free Ringer for an additional 60 min at 37°C for complete deesterification of the dye. Fura-2 fluorescence emission was monitored in a Shimadzu spectrofluorimeter at 505 nm from cells alternatively stimulated at 340 and 380 nm, using slits at 5 nm. Ionomycin 50 μM (or digitonin 0.05% v/v) and EGTA 10 mM were used for calibration. Intracellular calcium was subsequently calculated using the method of Grynkiewicz *et al.* [8]:

$$Ca^{2+}(nM) = K'_d \times (R - R_{min})/(R_{max} - R) \times F_0380/F_s380$$

with K'_d = Fura 2 dissociation constant at 37°C (= 224 nm); R = 340/380 nm fluorescence ratio; R_{max} = fluorescence ratio in high cytosolic calcium; R_{min} = fluorescence ratio in low cytosolic calcium; and F_0380/F_s380 = ratio of fluorescences at 380 nm in low calcium to that of high calcium.

Autofluorescence of nonloaded cells measured under the same experimental conditions were subtracted before computation of fluorescence ratios for calculation of cytosolic free Ca^{2+}.

PKC Activity

Protein kinase C (PKC) activity was assayed on macrophage homogenate according to a previously published method [9]. Briefly, cells were disrupted by sonication and PKC activity was assayed in triplicate by measuring ^{32}P transferred from [γ-^{32}P] to histone III-S (for details, see [9]).

Solutions and Reagents

BCECF-AM, Fura 2-AM and bis-oxonol $DiSBAC_2(3)$ were purchased from Molecular Probes (Eugene, OR). Fenspiride (8-(2-phenylethyl)-1-oxa-3,8-diazaspiro[4.5] decan-2-one) hydrochloride was obtained from Servier Laboratories (Suresnes, France). All other chemicals were purchased from Sigma-Coger (Paris, France). Fenspiride hydrochloride was diluted in water. Ionomycin, H-7 (1-(5-isoquinoline-sulfonyl)-2-methylpiperazine), staurosporine, PMA (phorbol 12-myristate 13-acetate), and Bay K8644 were added from freshly prepared concentrated stock solutions in DMSO (<0.2% v/v). Gramicidine D was dissolved in ethanol. PAF (platelet-activating factor) was used in presence of 0.1 mg/mL albumin (preliminary experiments showed that albumin had no effect on macrophage cytosolic calcium concentration).

Statistical Analysis

Values are given as mean ± SEM of n experiments. Differences in mean values were tested by using an unpaired Student's t-test.

RESULTS

Effect of Fenspiride on Agonist-Induced Calcium Signals

In resting alveolar macrophages, cytosolic free calcium was 120 ± 6 nM (n = 42). Fenspiride (0.1 to 1.2 mM) had no significant effect on basal calcium content, even after preincubation for 45 min with the compound (data not shown).

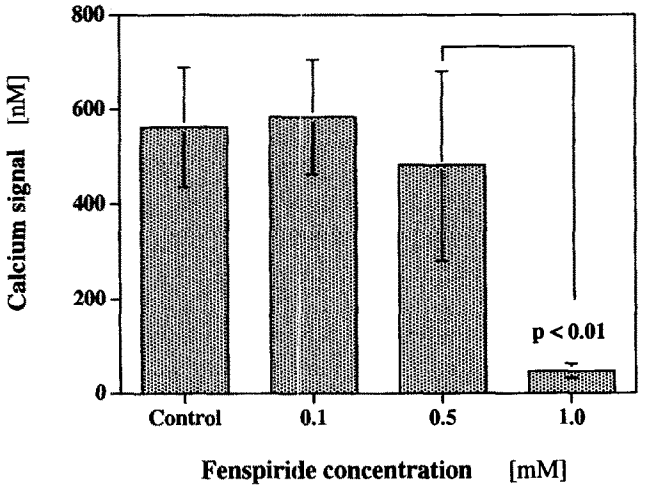

FIG. 1. Inhibition by fenspiride of the calcium signal elicited by Bay K8644 in rat alveolar macrophages. High fenspiride concentrations were required to reduce the calcium signal. Values are given as mean ± SEM (n = 5–9). Bay K8644 was used at 10 μM, a concentration that increased cytosolic free Ca^{2+} contents by 562 ± 127 nM (n = 3).

Fenspiride was tested in dose–response curves for its antagonistic properties against the calcium signals (ΔCa) evoked by the Bay K8644 compound (10 μM), ionomycin (1 μM), PAF (10 μM), and fMLP (1 μM). The calcium agonist Bay K8644 induced a control ΔCa equal to 562 ± 127 nM ($P < 0.05$ versus baseline, n = 3); Fig. 1 shows that high fenspiride concentrations (1 mM) were required to inhibit the Bay K8644-dependent calcium signal. Similarly, 1 mM fenspiride inhibited (i) 53 ± 16% ($P < 0.05$, n = 3) of fMLP-induced ΔCa (control = 249 ± 90 nM, n = 3), and (ii) 53 ± 18% ($P < 0.06$, n = 3) of ionomycin-induced ΔCa (control = 289 ± 85 nM, n = 3). Conversely, 1 mM fenspiride was unable to significantly affect the control calcium increases induced by PAF ($\Delta Ca_{(PAF)}$ = 509 ± 84 nM vs. 319 ± 106 nM, n = 6).

Fenspiride and PMA-Dependent pH Signal

In resting macrophages, basal pH_i was 7.16 ± 0.09 (in nominal absence of bicarbonate and at external pH = 7.38 to 7.42; n = 51). Cells were acidified to pH = 6.56 ± 0.19 (n = 17) by using the ammonium pulse method. Amiloride (1 mM) inhibited 60–90% of the cell pH recovery that followed cell acidification. The rate of amiloride-sensitive cytosolic alkalinization was equated to the rate of Na^+:H^+ exchange activity. In 17 experiments, Na^+:H^+ exchange activity was 0.070 ± 0.008 pH unit/min.

Figure 2 (upper panel) shows that PMA-stimulated Na^+:H^+ exchange in alveolar macrophages. A maximal 20–35% stimulation was obtained at 1 μM PMA (Fig. 2, upper panel). Interestingly, fenspiride (100 μM) was able to fully suppress PMA-stimulated Na^+:H^+ exchange activity (Fig. 2, upper panel).

We examined the action of two protein kinase inhibitors, H-7 and the microbial antifungal alkaloid staurosporine. Figure 2 (middle panel) shows the effect of H-7 (100 μM) and fenspiride (100 μM), alone or in combination, on Na^+:H^+ exchange activity in the presence of 1 μM PMA. It can be seen that all three conditions inhibited Na^+:H^+ exchange activity to a similar extent. Similar results were obtained with staurosporine (Fig. 2, lower panel).

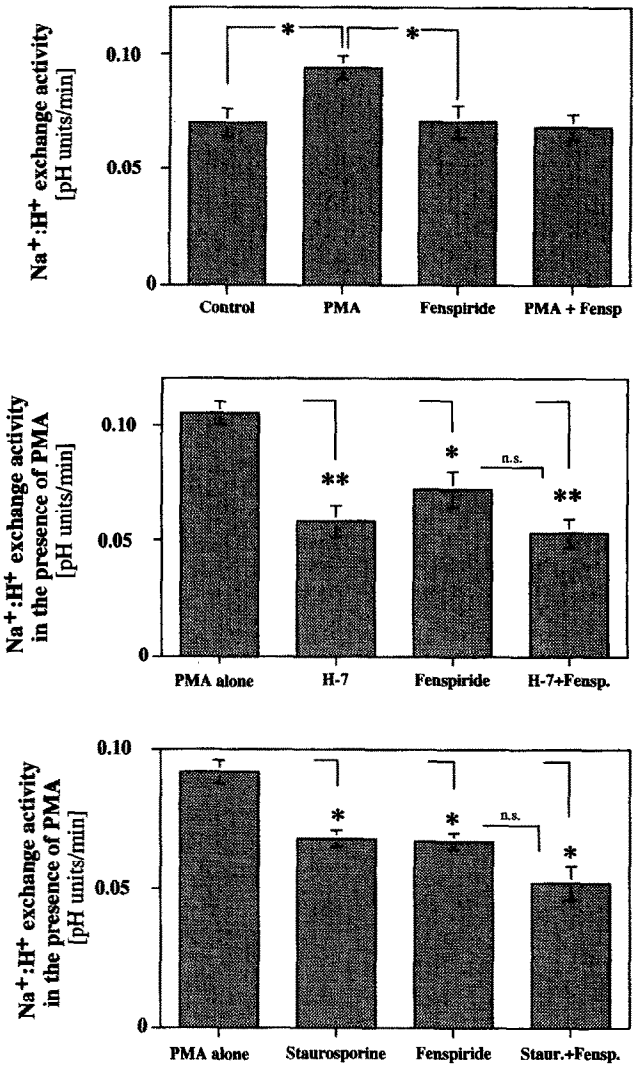

FIG. 2. Inhibition by fenspiride of PMA-mediated stimulation of Na^+:H^+ antiport in rat alveolar macrophages. Values are given as mean ± SEM (n = 3–5). Upper panel: full inhibition by 100 μM fenspiride of PMA-stimulated Na^+:H^+ exchange (PMA = 1 μM). Middle panel: effect of the protein kinase C inhibitor H7 (100 μM) and fenspiride (100 μM) on Na^+:H^+ exchange activity in the presence of 1 μM PMA. Both compounds, alone or in combination, inhibited the Na^+:H^+ antiport to a similar extent. Lower panel: effect of the protein kinase C inhibitor staurosporine (2 μM) and fenspiride (40 μM) on Na^+:H^+ exchange activity in the presence of 1 μM PMA. Both compounds, alone or in combination, inhibited the Na^+:H^+ antiport to a similar extent. *$P < 0.05$; **$P < 0.01$.

Figure 3 shows the effect of fenspiride on both fMLP- and PMA-stimulated Na^+:H^+ exchange activity. It can be seen that low concentrations of the compound fully inhibited the activation of Na^+-H^+ exchange induced by 50 nM fMLP (IC_{50} = 3.1 ± 1.9 nM) and by PMA (IC_{50} = 9.2 ±

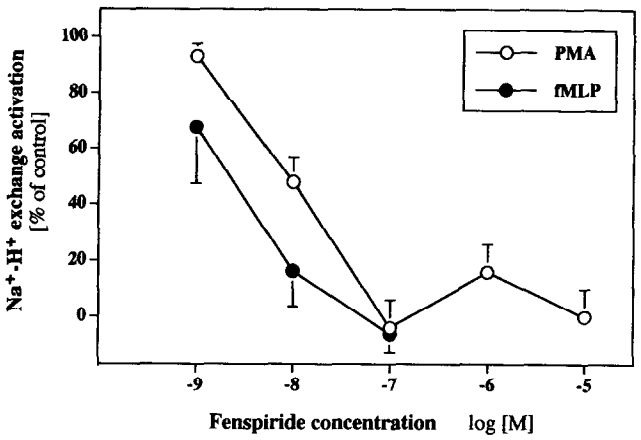

FIG. 3. Inhibition by fenspiride of PMA- and fMLP-mediated stimulation of Na^+:H^+ antiport in rat alveolar macrophages. PMA was used at 1 μM; fMLP was used at 50 nM. Values are given as mean ± SEM (n = 3–5). It can be seen that nanomolar concentrations of fenspiride fully inhibited PMA- and fMLP-mediated stimulation of Na^+:H^+ antiport.

3.1 nM); the difference between the IC_{50}s of fMLP and PMA were not significant. It is important to mention that these fenspiride concentrations was unable to modify basal Na^+:H^+ exchange activity (not stimulated by fMLP or PMA). Indeed, high doses of fenspiride (1 mM) were required to obtain a slight inhibition (30 ± 14%, $P < 0.05$, $n = 3$) of basal Na^+:H^+ exchange activity (i.e. not stimulated by fMLP or PMA).

PKC Activity

Fenspiride was unable to significantly inhibit the PKC activity of macrophage homogenates. Indeed, a slight, although not significant activation was found at 10 μM fenspiride (PKC activity = 3.50 ± 2.20 vs. 2.37 ± 1.61 pmol/10^6 cells/min in controls, $n = 3$) and at 100 μM fenspiride (PKC activity = 2.80 ± 1.71 vs. 2.57 ± 1.60 pmol/10^6 cells/min in controls, $n = 3$).

Effect of Fenspiride on Cell Membrane Depolarization

Fenspiride tested from 0.1 to 1 mM had no significant effect on basal values of the $DiSBAC_2(3)$ fluorescence signal of resting macrophages. Addition of gramicidine D (1 μg/mL) induced an increase in $DiSBAC_2(3)$ fluorescence, which was inhibited by 36 ± 3% ($P < 0.001$, $n = 8$) in the presence of 1 mM fenspiride.

DISCUSSION

Pipy *et al.* [7] have previously shown that fenspiride antagonizes the calcium signal induced by fMLP in peritoneal macrophages. However, this antagonistic action required very high fenspiride concentrations (IC_{50} = 1.1 mM, [7]). Here, we confirmed that millimolar concentrations of fenspiride were required to half-inhibit fMLP-induced calcium signal in rat alveolar macrophages. Such high fenspiride concentrations also inhibited the calcium signals induced by the slow calcium channel opener Bay 8644 and by ionomycin. Finally, fenspiride was unable to antagonize the PAF-induced calcium signal: this lack of effect cannot be explained by the magnitude of the increase in cytosolic calcium, because Bay K8644 increased calcium to a similar extent as Bay 8644 and ionomycin. The explanation should perhaps be sought in specific aspects of the mechanism of action of PAF.

The above antagonistic actions on calcium signals required much more than therapeutic fenspiride concentrations, which are in the micromolar range [10]. These rather negative results concerning the calcium signal suggested that fenspiride was probably acting on another signalling pathway. On the other hand, protein kinase C is known to stimulate Na^+:H^+ antiport activity and increase the calcium sensitivity of a number of cellular processes [11, 12]. Therefore, we explored the action of fenspiride on PMA- and fMLP-mediated stimulation of the antiport.

Fenspiride potently antagonized PMA- and fMLP-mediated stimulation of the antiport in rat alveolar macrophages (with similar inhibitory constants). This stimulatory action was observed in the nanomolar range of fenspiride concentrations and was not due to a direct effect on the Na^+:H^+ antiport protein. In the presence of 40–100 μM fenspiride (and PMA), the PKC inhibitors H-7 and staurosporine produced no further inhibition of Na^+:H^+ antiport, further suggesting that fenspiride was acting on the protein kinase C signaling pathway.

In spite of the above results, fenspiride was unable to significantly inhibit the phosphorylating PKC activity of macrophage homogenate, even at high concentrations (100 μM). However, it remains possible that fenspiride is able to inhibit PKC translocation or act via an unusual mechanism of agonist-mediated stimulation of the antiport. In this respect, it is important to recall that in mouse peritoneal macrophages, PKC can act via MAP kinase (mitogen-activated protein kinase) activation by a complex mechanism, which depends on the agonist used [13]. Moreover, agonist-dependent stimulation of the Na^+:H^+ antiport can take place by a PKC-independent pathway [14]. Therefore, the mechanism of action of fenspiride on agonist-dependent antiport stimulation deserves further investigation. The potential interest of this approach to the antiinflammatory mechanism of fenspiride is illustrated by the fact that the sodium-proton antiport inhibitor amiloride possesses antiinflammatory properties in mice [15].

Regarding membrane potential, fenspiride was unable to modify the basal bis-oxonol $DiSBAC_2(3)$ fluorescence signal, a marker of the resting potential of macrophage cell membranes (which is close to −50 mV, [16]). Conversely, 1 mM fenspiride reduced the increase in bis-oxonol $DiSBAC_2(3)$ fluorescence signal induced by gramicidine D (1 μg/mL) by 36%.

As shown by Kremer *et al.* [17, 18], gramicidine can depolarize cell membranes up to 0 mV. Therefore, fen-

spiride should induce a shift of ≈18 mV in the gramicidine D-dependent cell membrane depolarization. Whether this phenomenon can explain part of the inhibition by fenspiride of the Bay K8644-mediated calcium signal (via voltage-dependent calcium channels) deserves further investigation. These studies should also determine whether fenspiride acts via some direct or indirect modification of cell membrane potassium or chloride conductances.

In conclusion, fenspiride inhibits the agonist-induced calcium signal in rat alveolar macrophages, but at concentrations far above therapeutic fenspiride levels. Conversely, low fenspiride concentrations inhibited fMLP- and PMA-mediated stimulation of the Na^+:H^+ antiport. This action could reduce the calcium sensitivity of macrophage cellular processes and participate in the antiinflammatory mechanism of fenspiride.

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