We have tested this set of tannins for their effects on the viability of stimulated normal human peripheral blood lymphocytes after 4 days of culture. The inactive compound GN-8 showed moderate toxicity (58% viable vs 94% for controls) at the tested concentration of 40 μM. The other inactive compound, GN-11 (20 μM), and all of the active compounds (10 μM) had no effect on the viability of the lymphocytes.

One approach in developing anti-HIV drugs is to look for compounds which could interfere with the interaction between viral rgp120 and cellular CD4. Tannins such as chebulinic acid and punicalin could be excellent lead compounds for further drug development.

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The effect of anion transport inhibitors and extracellular Cl^- concentration on eosinophil respiratory burst activity

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Abstract—Furosemide has been shown recently to protect asthmatic patients against certain bronchoconstrictor challenges. We investigated the effect of furosemide on eosinophil function. Since furosemide may be exerting its inhibitory effect on the eosinophil by inhibiting anion transport, we also assessed the effects of the anion transport inhibitors 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). Furosemide, NPPB and DIDS inhibited the eosinophil respiratory burst in response to leukotriene B_4 (LTB_4) and, to a smaller extent, inhibited the response to opsonized zymosan (OZ). To assess whether the anion transport inhibitors were achieving their inhibitory effect by inhibiting an influx of Cl^- ions into the eosinophil, the effect of removing extracellular Cl^- on eosinophil function was determined. OZ-induced H_2O_2 production was inhibited by removing extracellular Cl^- whereas the LTB_4 response was not affected by the concentration of extracellular Cl^-.

Recently, it has been shown that the loop diuretic furosemide protects asthmatic patients against various indirect bronchoconstrictor challenges. Inhalation of furosemide was demonstrated to inhibit the airway response to exercise [1], distilled water [2], metabisulphite [3] and adenosine [4], and to inhibit the early and late response to antigen [5]. The mechanisms of action of furosemide on the asthmatic response are yet to be established. One possibility is that furosemide may exert an inhibitory effect on inflammatory cells which may be activated by these challenges. Eosinophils are implicated in a range of allergic and inflammatory disorders, and can cause tissue injury by the generation of active oxygen species (e.g. superoxide anions and H_2O_2) via an oxidative burst, and by the release of toxic proteins [6].

In the present study, we have therefore investigated the effect of furosemide on the respiratory burst of the eosinophil using a continuous assay for H_2O_2. Furosemide may exert an effect on the eosinophil by inhibiting anion transport and we therefore examined the effects of the Cl^- transport inhibitors 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB*) [7] and 4,4′-diisothiocyanatostilbene-

* Abbreviations: DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; LTB_4, leukotriene B_4; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid; OZ, opsonized zymosan.

2,2'-dilsulfonic acid (DIDS) [8] on the respiratory burst. In addition, the effects of removing extracellular Cl^- on eosinophil function were examined.

Materials and Methods

Percoll was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Hanks' balanced salt solution was from Flow Laboratories Ltd (Rickmansworth, U.K.). NPPB and leukotriene B_4 (LTB_4) were gifts from Hoechst A.G. (Frankfurt, Germany) and Bayer U.K. (Stoke Poges, U.K.), respectively. Zymosan, furosemide, DIDS and reagents and buffers were from the Sigma Chemical Co. (Poole, U.K.). Opsonized zymosan (OZ) was prepared by incubating zymosan in Hanks' balanced salt solution containing 20% guinea pig serum for 20 min at 37°.

Eosinophils were obtained from human serum-treated guinea pigs by weekly peritoneal lavage and purified using a discontinuous density gradient of isomolar Percoll solutions as described previously [9]. Eosinophils with a purity of >95% were resuspended at 10^7 cells/mL in Ca^{2+}-free Hepes buffer (138 mM NaCl, 6 mM KCl, 1 mM NaH_2PO_4, 10 mM Hepes, 5 mM $NaHCO_3$ and 5.5 mM glucose). For Cl^--free buffer, NaCl and KCl were replaced by equimolar volumes of their respective gluconate salts.

The generation of H_2O_2 by eosinophils was measured using a fluorometric assay as described previously [10]. Following addition of 1 mM $MgSO_4$ and 1 mM $CaCl_2$ (1 mM calcium gluconate for Cl^- free buffer), the cells were incubated at 37° for 3 min prior to stimulation by 0.5 mg/mL OZ or 100 nM LTB_4. The rate of decrease of the fluorescence was measured and converted to rate of H_2O_2 production by comparison with a standard curve for known H_2O_2 concentrations.

Results are presented as mean values ± SEM. The differences between the values obtained in the absence and presence of drug were tested for significance by a two-tailed Student's *t*-test for paired observations.

Results and Discussion

Preliminary experiments indicated that 10^{-7} M LTB_4 and 0.5 mg/mL OZ were submaximal concentrations of these stimuli (data not shown). LTB_4 (10^{-7} M) stimulated a rapid and transient production of H_2O_2 with a peak rate of 4.92 ± 0.48 nmol/min. OZ (0.5 mg/mL) produced a release of H_2O_2 which was slower in onset but more sustained than that produced by LTB_4. The peak rate of H_2O_2 generation produced by OZ was 2.07 ± 0.19 nmol/min.

Furosemide, NPPB and DIDS, at the concentrations used in these experiments, did not interfere with the measurement of H_2O_2. Furosemide caused a concentration-dependent inhibition of LTB_4-induced H_2O_2 generation with an IC_{50} value of $2.70 \pm 0.29 \times 10^{-5}$ M (Fig. 1). In contrast, furosemide (10^{-4} M) only inhibited the OZ response by 25.2 ± 2.90% ($P < 0.01$).

Furosemide is known to inhibit the $Na^+/K^+/2Cl^-$ cotransporter system in a number of tissues [11]. Although high concentrations of furosemide were required in our study, they were comparable with concentrations used to inhibit the $Na^+/K^+/2Cl^-$ cotransporter protein on the human erythrocyte [12], and with concentrations required to inhibit airway nerves in guinea pig bronchi [13].

In addition to inhibition of the $Na^+/K^+/2Cl^-$ cotransporter, high concentrations of furosemide have been shown to inhibit Cl^- channels of the rat lacrimal gland [8] and non-selective cation channels in the rat exocrine pancreas [8]. Thus, the inhibitory effect of furosemide on the eosinophil cannot be ascribed to a particular channel mechanism. We therefore proceeded to investigate the effects of examples of two other classes of Cl^- channel blockers, DIDS, a stilbene disulfonate, and NPPB, a derivative of diphenylamine-2-carboxylic acid.

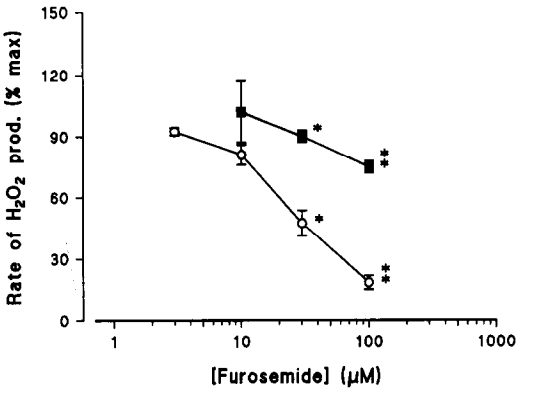

Fig. 1. Effect of furosemide on the rate of H_2O_2 generation stimulated by 10^{-7} M LTB_4 (○) and 0.5 mg/mL OZ (■). The results expressed as a % of the maximum rate of H_2O_2 produced in the absence of furosemide represent the mean ± SEM of four experiments. The differences between the values obtained in the absence and presence of furosemide were tested for significance using the Student's *t*-test. P values: * < 0.05; ** < 0.01; *** < 0.001.

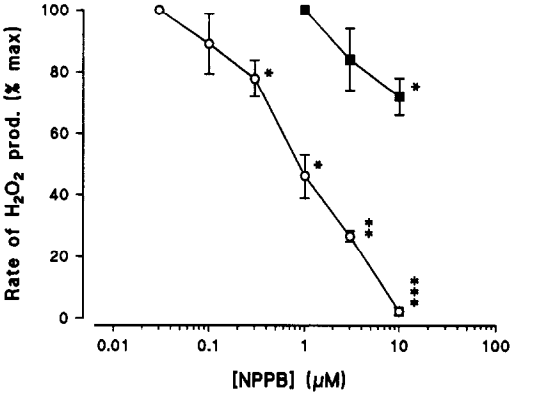

Fig. 2. Effect of NPPB on the rate of H_2O_2 generation stimulated by 10^{-7} M LTB_4 (○) and 0.5 mg/mL OZ (■). The results expressed as a % of the maximum rate of H_2O_2 produced in the absence of NPPB represent the mean ± SEM of four experiments.

NPPB and DIDS each caused a concentration-dependent inhibition of LTB_4-induced H_2O_2 generation with IC_{50} values of $9.20 \pm 2.0 \times 10^{-7}$ M and $5.5 \pm 3.20 \times 10^{-7}$ M, respectively (Figs 2 and 3). NPPB and DIDS also inhibited the OZ-induced H_2O_2 production but to a much lesser extent than the LTB_4 response. NPPB (10^{-5} M) and DIDS (3×10^{-6} M) inhibited the OZ response by 28.5 ± 5.9% ($P < 0.05$) and 29.0 ± 6.5% ($P < 0.05$), respectively.

DIDS has been shown to block the small and large conductance Cl^- channels in rabbit urinary bladder [14], but is not selective, as it also inhibits the anion-exchange system in red blood cells [15]. NPPB inhibits Cl^- channels on the thick ascending limb of the loop of Henle, and other epithelial Cl^- channels [7]. The specificity of NPPB with respect to other channel types has still to be determined. None of these anion transport inhibitors is sufficiently

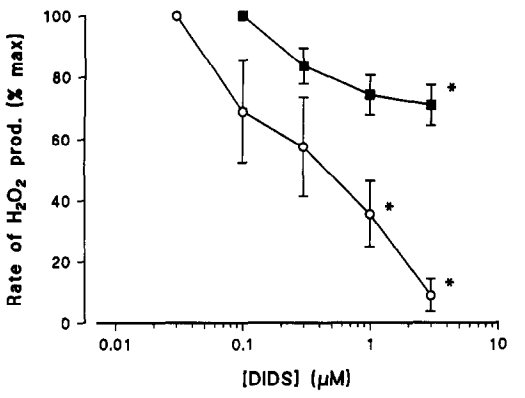

Fig. 3. Effects of DIDS on the rate of H_2O_2 generation stimulated by 10^{-7} M LTB_4 (○) and 0.5 mg/mL OZ (■). The results expressed as a % of the maximum rate of H_2O_2 produced in the absence of DIDS represent the mean ± SEM of four experiments.

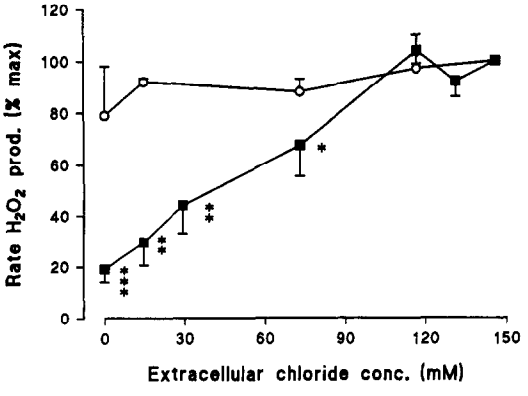

Fig. 4. Effect of extracellular Cl^- concentration on the rate of H_2O_2 generation stimulated by 10^{-7} M LTB_4 (○) and 0.5 mg/mL OZ (■). The results expressed as a % of the maximum rate of H_2O_2 produced in the presence of normal extracellular Cl^- (146 mM) represent the mean ± SEM of seven experiments.

selective for us to state that a Cl^- flux is involved in the oxidative burst of the eosinophil. However, since furosemide, NPPB and DIDS all inhibit Cl^- channels, albeit in a number of different tissues, it is possible that they may be inhibiting an influx of Cl^- ions in the eosinophil, necessary for the LTB_4-induced respiratory burst, but not so critical for the OZ response. We thus investigated the effect of Cl^- replacement on eosinophil respiratory burst.

Eosinophils were bathed in a Hepes buffer in which the Cl^- ions had been replaced with the impermeant anion gluconate. OZ-induced H_2O_2 generation was observed to be dependent on the concentration of extracellular Cl^- (Fig. 4). In the absence of extracellular Cl^-, the OZ response was inhibited by 80.8 ± 5.0% ($P < 0.001$) and the production of H_2O_2 recovered as the concentration of extracellular Cl^- was increased. In contrast, the LTB_4 response was independent of the extracellular Cl^- concentration, indicating that the anion transport inhibitors were not inhibiting the LTB_4-induced H_2O_2 production in the eosinophil by inhibiting an influx of Cl^- ions.

The finding that the responses stimulated by OZ and LTB_4 were affected to very different extents by the anion transport inhibitors and the extracellular Cl^- concentration suggests that these two stimuli activate the respiratory burst through two different mechanisms. The OZ response had a requirement for extracellular Cl^- but was relatively unaffected by the anion transport inhibitors, whereas the LTB_4 response did not depend on extracellular Cl^- ions but was inhibited by furosemide, NPPB and DIDS. The anion transport inhibitors may be achieving their inhibitory effect on the LTB_4 response by inhibiting an efflux of Cl^- ions, or an influx or efflux of other anions. Anion flux experiments are thus required to investigate this further as, at present, no data exist on ion channels in the eosinophil. Furthermore, other eosinophil stimuli may have different ion requirements to either LTB_4 or OZ.

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Stimulation of insulin secretion by glucose in the absence of diminished potassium ($^{86}Rb^+$) permeability

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Abstract—Two inhibitors of the nucleotide-sensitive K^+ (K_{ATP}) channel, tolbutamide and quinine, were utilized in order to assess the role of this channel in glucose-stimulated insulin release from perifused rat islets. In the absence of these drugs, the addition of 15 mM glucose elicited a marked biphasic stimulation of insulin secretion concomitant with a reduction in the rate of $^{86}Rb^+$ efflux. In the presence of either 500 μM tolbutamide or 100 μM quinine, a reduced rate of efflux of $^{86}Rb^+$ was observed together with an elevated rate of insulin release. Under such conditions, the addition of 15 mM glucose retained the ability to stimulate insulin secretion though this was associated with a marked increase in $^{86}Rb^+$ efflux. It is concluded that a net reduction in β-cell K^+ permeability is not an obligatory step in glucose-stimulated insulin release. Thus, glucose is likely to exert depolarizing actions on the β-cell in addition to the closure of K^+ channels.

The stimulation of insulin secretion by glucose and other nutrient secretagogues depends upon the metabolism of the nutrient within the β-cell [1] and the subsequent depolarization of the β-cell plasma membrane (for reviews, see Refs 2 and 3). It is currently believed that a rise in the cytosolic ATP/ADP ratio resulting in the closure of nucleotide-sensitive K^+ (K_{ATP}) channels provides the link between nutrient metabolism and plasma membrane depolarization [3, 4]. This channel also appears to be at least one of the sites of action of the hypoglycaemic sulphonylureas and quinine, both of which have been demonstrated to cause channel closure [5, 6].

We have utilized this particular action of these drugs in order to ascertain whether the insulinotropic effect of glucose persists under conditions of pharmacological inhibition of the K_{ATP} channel.

Materials and Methods

Islets were isolated by collagenase digestion from adult Wistar rats, and perifused at a rate of 1 mL/min with a physiological saline medium consisting of NaCl (110 mmol/L), KCl (5 mmol/L), $MgCl_2$ (1 mmol/L), $CaCl_2$ (1 mmol/L), $NaHCO_3$- (25 mmol/L) and HEPES (20 mmol/L), gassed with O_2/CO_2 (19:1 v/v). $^{86}Rb^+$ efflux and insulin release were measured in groups of 100 and 25 islets, respectively, as described elsewhere [7].

$^{86}Rb^+$ (4 mCi/mg) was obtained from the Radiochemical Centre (Amersham, U.K.). Tolbutamide and quinine were purchased from the Sigma Chemical Co. (Poole, U.K.). Analysis of statistical significance, utilizing the Student's *t*-test, was performed by comparing overall $^{86}Rb^+$ efflux or insulin secretory rates between the 30th and 35th min of perifusion with the corresponding rates between the 40th and 45th min.

Results and Discussion

The addition of 15 mM glucose to perifused rat islets resulted in a rapid reduction in the rate of efflux of $^{86}Rb^+$ from preloaded islets (Fig. 1A; $P < 0.001$), confirming previous reports [8, 9]. It is currently believed that this effect represents a reduction in plasma membrane K^+ permeability resulting from closure of K_{ATP} channels [3, 4] and accounts for the depolarization of the β-cell plasma membrane observed in response to the sugar [10]. This depolarization is thought to result in the opening of voltage-sensitive calcium channels [11] and calcium entry into the cell which triggers insulin release [12]. The accompanying biphasic stimulation of insulin release by glucose ($P < 0.001$) is shown in Fig. 2A.

Perifusion of islets in the presence of supramaximal concentrations of either tolbutamide (500 μM; Fig. 1B) or quinine (100 μM; Fig. 2C) resulted in a reduced fractional outflow rate of $^{86}Rb^+$ ($P < 0.001$ in both cases), presumably reflecting inhibition of K_{ATP} channels by these compounds [5, 6]. In the presence of either drug, the subsequent addition of 15 mM glucose caused a pronounced increase in the rate of efflux of $^{86}Rb^+$ (Fig. 1B and C; $P < 0.002$ and 0.01, respectively). In contrast to a previous report [13], this increase was not preceded by a transient reduction in $^{86}Rb^+$ outflow rate in the presence of quinine in response to glucose. The underlying mechanisms to this apparent increase in net K^+ permeability are uncertain, although this effect is reminiscent of the enhanced rate of $^{86}Rb^+$ efflux observed by Carpinelli and Malaisse [14] upon raising the glucose concentration from 8.3 mM to higher concentrations. These authors proposed that increased cytosolic [Ca^{2+}] may be responsible for this glucose-stimulated increase in K^+ permeability. An increase in