Inhibition of inositol 1,4,5-trisphosphate 5-phosphatase by micromolar concentrations of disulfiram and its analogues

Christopher J. FOWLER,*†§ Gunilla BRÄNNSTRÖM,*† P. Charlotte AHLGREN,* Lennart FLORVALL* and Karl E. O. ÅKERMAN†

* Astra Arcus AB, CNS Preclinical R & D, S-151 85 Södertälje, Sweden, and † Department of Biochemistry and Pharmacy, Åbo Academy, SF-20500 Turku, Finland

Following a preincubation period of 10 min, disulfiram and its analogues FLA 46, FLA 63, FLA 99, EWP 815 and EWP 840 inhibited the breakdown of 10 μM [^3H]Ins(1,4,5) P_3 by Ins(1,4,5) P_3 5-phosphatase from GH $_3$ cells, with IC $_{50}$ values (in μM), for soluble/particulate enzymes respectively, of: disulfiram, 24/24; FLA 46, 23/30; FLA 63, 24/6; FLA 99, 50/48; EWP 815, 8/6; EWP 840, 11/8. The inhibition produced by FLA 99 was time-dependent in nature, although inhibition was found in the absence of a preincubation period. EWP 815 and EWP 840 were more potent inhibitors of Ins(1,4) P_2 phosphatase than of Ins(1,4,5) P_3 5-phosphatase. Thyrotropin-releasing hormone (TRH; 3/100 μM)-stimulated inositol phospholipid breakdown in prelabelled GH $_3$ cells was inhibited by disulfiram (IC $_{50}$ values 63/52 μM respectively), FLA 46 (89/110 μM ,

EWP 815 (83/71 μM) and EWP 840 (220/200 μM), without affecting basal breakdown rates. FLA 99 did not inhibit either basal or TRH-stimulated activity at any of the concentrations tested (30, 100 and 300 μM). [^3H]Ins(1,4,5) P_3 binding to its cerebellar receptor was not inhibited by any of the compounds over a concentration range of 3–300 μM , although an increased level of binding was seen at high concentrations. FLA 99 and EWP 840 increased the basal intracellular Ca $^{2+}$ concentration in GH $_3$ cells, but with no corresponding effect on the Ca $^{2+}$ response to TRH stimulation. These compounds did not increase the cellular permeability to Trypan Blue, but did affect cell proliferation. It is concluded that disulfiram and related compounds produce dramatic effects on Ins(1,4,5) P_3 metabolism in GH $_3$ cells.

INTRODUCTION

It is now well established that the intracellular second messenger Ins(1,4,5) P_3 , generated by the receptor-coupled phosphodiesteratic cleavage of PtdIns(4,5) P_2 , acts upon specific receptors to mobilize Ca $^{2+}$ from intracellular stores (for a review, see Berridge and Irvine, 1989). Both Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 , which is produced from Ins(1,4,5) P_3 by the action of a 3-kinase and which acts synergistically with Ins(1,4,5) P_3 (see Irvine, 1990), are metabolized by a class of Ins(1,4,5) P_3 5-phosphatase enzymes, which can be both soluble and particulate in nature (for a review, see Shears, 1989).

Recently it was reported that the thiol reagents thimerosal and oxidized glutathione affect Ca $^{2+}$ signalling in hepatocytes (Missiaen et al., 1991). Little is known about the effects of such reagents on the metabolism of Ins(1,4,5) P_3 , although potent inhibition of the 5-phosphatase enzyme by *p*-chloromercuribenzoate has been reported (Erneux et al., 1986). The anti-(alcohol abuse) drug disulfiram and its analogues are known to block thiol groups (see Lindahl & Åkerström, 1965), which would suggest that such compounds may affect Ca $^{2+}$ signalling mediated by the phosphoinositide cycle. This possibility has been investigated in the present study. As a model system, we have used GH $_3$ rat pituitary cells, which express thyrotropin-releasing hormone (TRH) receptors. The stimulation of these receptors mobilizes Ca $^{2+}$, the rapid phase of which is mediated via production of Ins(1,4,5) P_3 (Mollard et al., 1990).

MATERIALS AND METHODS

Materials

Rat GH $_3$ pituitary cells (ATCC no. CCL 82.1) were obtained from Flow Laboratories, Irvine, Scotland, U.K., and were used at passages 37–45. *D*-myo-[2- ^3H]Ins(1,4,5) P_3 (specific radioactivity 1 Ci/mmol), *myo*-[2- ^3H]inositol ([^3H]Ins; specific radioactivity 21.6 Ci/mmol, with added PT6-271 to absorb breakdown products), non-radioactive Ins(1,4,5) P_3 and Amprep SAX (100 mg) minicolumns were obtained from Amersham International, Amersham, Bucks., U.K. The molecular mass of the non-radioactive Ins(1,4,5) P_3 was taken to be that of the free acid (420 Da). The NENQUEST drug discovery kit for the Ins(1,4,5) P_3 receptor was obtained from Du Pont-de Nemours (Deutschland) G.m.b.H., Biotechnology Systems Division, Bad Homburg, Germany ([^3H]Ins(1,4,5) P_3 specific radioactivity 17 Ci/mmol). Fura-2/AM was obtained from Molecular Probes Inc., Eugene, OR, U.S.A. TRH (approx. 95% pure), glucose 6-phosphate, disulfiram and heparin (low-molecular-mass from porcine intestinal mucosa; sodium salt) were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. L-Cystine was obtained from BDH. The disulfiram analogues FLA 46, FLA 49, FLA 63, FLA 99, EWP 815 and EWP 840, the dithiocarbamic acid FLA 57 and the dithiocarbamic acid ethyl ester FLA 108 were synthesized at Astra (see Florvall and Corrodi, 1970). Ham's F-10 medium was obtained from Flow Laboratories, and was supplemented with 10 mM Hepes, 2 mM glutamine,

Abbreviations used: TRH, thyrotropin-releasing hormone; DMSO, dimethyl sulphoxide; HBSS, Hanks' balanced salt solution; [Ca $^{2+}$] $_i$, intracellular Ca $^{2+}$ concentration.

† Present address: Astra Pain Control AB, S-151 85 Södertälje, Sweden.

§ To whom correspondence should be addressed.

100 µg/ml streptomycin sulphate (Sigma) and 70 µg/ml benzyl penicillin K (Astra). Foetal calf serum was obtained from Gibco. Disulfiram and analogues were dissolved in dimethyl sulphoxide (DMSO) (final concentration in assay 0.5–2%), whereas the other compounds were dissolved in distilled water. Assay controls contained the same DMSO concentration as the test samples.

Ins(1,4,5) P_3 5-phosphatase assay

The assay used has been described in detail by Fowler and Brännström (1990). Briefly, GH₃ cells were cultured in 175 cm² flasks in supplemented Ham's F10 medium containing 10% foetal calf serum, and harvested when 50% confluency was reached. After washing with ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.38 mM Na₂HPO₄ and 1.47 mM KH₂PO₄), the cells were detached by addition of PBS/0.02% EDTA. After centrifugation at 1000 *g* for 4 min and resuspension in 20 mM Tris/HCl containing 0.2 mM EDTA, pH 7.6, the cells, in a volume of 300–350 µl/flask, were left on ice for 10 min and then homogenized using a Dounce homogenizer (20 strokes). The homogenate was centrifuged at 100 000 *g* for 60 min to give a supernatant (soluble fraction) and a pellet (particulate fraction). The pellet was resuspended in 20 mM Tris/HCl/0.2 mM EDTA, pH 7.6, to the same volume as the supernatant, and aliquots of both fractions were frozen at –70 or –20 °C until used for assay.

Aliquots (20 µl) of the soluble (40–76 µg of protein) or particulate (85–163 µg of protein) fractions were added to 200 µl of assay buffer (10 mM Hepes, 10 mM KCl, 4 mM MgCl₂ and 1 mM EGTA, pH 7.2) containing the test compounds and incubated for 10 min at 37 °C in 2 ml Sarstedt tubes. The tubes were thereafter placed on ice. [³H]Ins(1,4,5) P_3 (30 µl; final concentration 10 µM unless otherwise stated; see Fig. 3) was added and the samples were incubated for 10 min at 37 °C. Blank values were determined in the presence of test compound but without enzyme source. Reactions were stopped by addition of 600 µl of ice-cold chloroform/methanol (1:2, v/v), and the samples were vortex-mixed and placed on ice. When all samples had been treated in this manner, they were vortex-mixed for 10 min using an SMI Model 2601 multi-tube vortexer (Scientific Manufacturing Industries, Emeryville, CA, U.S.A.). Distilled water (750 µl) was added and the samples were vortex-mixed for 60 s followed by centrifugation to separate the phases.

The ³H-labelled inositol phosphates were separated using Amprep SAX (100 mg) minicolumns and elution conditions described previously (Fowler & Brännström, 1990). The total ³H eluted as [³H]Ins(1,4) P_2 , [³H]Ins P_1 and *myo*-[³H]inositol was expressed as a fraction of the ³H added to the assays, and the activities in the presence of the test compounds were expressed as percentages of the control

Assay of reversibility of inhibition of particulate Ins(1,4,5) P_3 5-phosphatase

Aliquots (765 µl) of particulate fractions in assay buffer were preincubated with the test compounds for 60 min at 37 °C. In each case, an aliquot (225 µl) was removed for assay of activity and the remaining 540 µl was centrifuged at 100 000 *g* for 60 min. The resultant pellets were resuspended in assay buffer to a volume of 540 µl and aliquots (225 µl) were assayed for activity. The mean yield of activity in the control samples was 55 ± 12%.

Basal and TRH-stimulated phosphoinositide hydrolysis in GH₃ cells

GH₃ cells were cultured in supplemented Ham's F-10 medium containing 10% foetal calf serum at split ratios of 1:8 in 75 cm² flasks and used between passages 43 and 44. After passage, cells (approx. 2 × 10⁵ in 3 ml of medium) were transferred to tissue culture cluster plates (6 wells/plate; well diameter 35 mm; Costar, Cambridge, MA, U.S.A.). After 4 days, medium was replaced by 2 ml of Ham's F-10 medium containing 10% foetal calf serum and *myo*-[³H]inositol (0.5 µCi/well) and incubated for approx. 17 h. The rates of basal and TRH-stimulated phosphoinositide hydrolysis were determined essentially as described for cultured cells by Fowler et al. (1991). Briefly, the *myo*-[³H]inositol-containing medium was removed and replaced with 2 ml of foetal-calf-serum-free medium containing LiCl (8 mM) and the test compound. After incubation for 30 min at 37 °C, TRH (0, 3 or 100 nM) was added and the cells were incubated for a further 30 min at 37 °C, whereafter the medium was aspirated and the cluster plates placed on ice. After addition of 0.5 ml of ice-cold methanol, the cells were scraped from the wells. These cell/methanol suspensions were then pipetted into 15 ml glass centrifuge tubes, the wells were rinsed with 0.5 ml of methanol and the methanol fractions were combined. Chloroform (1 ml) and distilled water (0.5 ml) were added, and the centrifuge tubes were vortex-mixed and then centrifuged (~2000 *g*) for 5 min to separate the phases. Aliquots of the methanol/water phase (750 µl, containing both labelled inositol phosphates and *myo*-[³H]inositol) and the chloroform phase (400 µl, containing the labelled phosphatidylinositol) were taken. The labelled inositol phosphates were separated from *myo*-[³H]inositol using an ion-exchange resin (Berridge et al., 1982) and the inositol phosphate radioactivity was expressed as a fraction of the total ³H incorporated (see Fowler et al., 1991). The degree of inhibition produced by the test compounds was then calculated.

Assay of [³H]Ins(1,4,5) P_3 receptor binding

The NENQUEST drug discovery kit for [³H]Ins(1,4,5) P_3 receptor binding was used. Briefly, test compounds (100 µl) were added to a mixture (provided by the manufacturers) consisting of [³H]Ins(1,4,5) P_3 (1.2 nM final concentration), a membrane preparation from bovine cerebellum containing the Ins(1,4,5) P_3 receptor, and assay buffer in a final volume of 500 µl. The samples were incubated for 60 min at 4 °C, after which the assay tubes were centrifuged for 10 min at 1000 *g* (4 °C). The pellets were solubilized in 50 µl of 0.15 M NaOH, and the radioactivity was determined by liquid scintillation spectroscopy.

Measurement of intracellular Ca²⁺ concentration ([Ca²⁺]_i)

The [Ca²⁺]_i in GH₃ cells was measured as described by Åkerman and Heikkilä (1990). Briefly, cells were detached from culture flasks (800 ml) by treatment with EDTA (0.02% in PBS) and thereafter washed in the Na⁺-based experimental medium [Hanks' balanced salt solution (HBSS)] containing (mmol/l): NaCl 137, KCl 5.0, MgCl₂ 1.2, KH₂PO₄ 0.44, NaHCO₃ 4.2, glucose 10, Tes 20 (pH 7.4). The cells were suspended in this medium at 37 °C, 2 µM Fura-2/AM and 1 mM CaCl₂ were added, and the cells were incubated for 30–40 min. Subsequently, the cells were sedimented by centrifugation, washed in HBSS, divided into separate Eppendorf tubes and resedimented. The medium was moved and the cells were stored on ice as pellets covered with a small amount of medium. Each experiment was

started by suspending one tube in HBSS containing 1 mM CaCl_2 , and the fluorescence was recorded at alternate 340 nm/380 nm excitation using a SPEX CM fluorescence spectrophotometer. The cells were constantly stirred using a magnetic stirring device. The signal was calibrated using 5 μM ionomycin and 10 mM EGTA to obtain the maximal and minimal fluorescence values respectively, and the $[\text{Ca}^{2+}]_i$ was calculated as described by Grynkiewicz et al. (1987).

Determination of cell density

Cell density determinations were undertaken for cells grown in 6-well tissue culture cluster plates, as described by Stenberg (1981). Briefly, after removal of medium, the cells were mildly trypsinized at 37 °C with trypsin/EDTA solution (Gibco). The suspensions were then diluted with particle-free buffer (Baker Chemicals, Denver, The Netherlands) and their cell density was determined using a multichannel electronic cell counter (Analysinstrument AB, Stockholm, Sweden).

Protein determinations

Protein contents of the samples were determined by the method of Markwell et al. (1978), using BSA as standard.

RESULTS

Effects of disulfiram and its analogues on soluble and particulate Ins(1,4,5) P_3 5-phosphatases in GH_3 cells

Disulfiram and its analogues FLA 46, FLA 63, FLA 99, EWP 815 and EWP 840 (for chemical structures, see Table 1)

Table 1 Structures of disulfiram and the disulfiram analogues investigated in the present study and their IC_{50} values towards $[\text{H}]\text{Ins}(1,4,5)P_3$ dephosphorylation in GH_3 cell preparations

IC_{50} values were calculated graphically from the mean data given in Tables 2 and 3 and Figure 1, using a 10 min preincubation phase. General formula:

Compound	R	IC_{50} (μM)	
		Soluble	Particulate
Disulfiram		24	24
FLA 46		23	30
FLA 63		24	6
FLA 99		50	48
EWP 815		8	6
EWP 840		11	8

inhibited the dephosphorylation of 10 μM $[\text{H}]\text{Ins}(1,4,5)P_3$ by both the particulate and soluble fractions of GH_3 cells. The individual values, following a 10 min preincubation phase, are given in Tables 2 and 3, and the IC_{50} values determined graphically from these data are given in Table 1. The compounds inhibited (with the possible exception of FLA 63) the particulate and soluble enzymes to about the same degree, with IC_{50} values in the range 6–50 μM , and with a rank order of potency (for the soluble enzyme) of EWP 815 $\approx$ EWP 840 > disulfiram $\approx$ FLA 46 $\approx$ FLA 63 > FLA 99 (Table 1). Given the time-dependence of inhibition (see below), the absolute order of potencies may vary with the preincubation time used. Nevertheless, the potencies of these compounds are in all cases very much greater than that of 2,3-bisphosphoglycerate (for comparison of this compound with disulfiram, see Figure 1).

Other analogues and compounds related to disulfiram were also tested. FLA 49, at a concentration of 100 μM , produced $\sim 30\%$ inhibition of 5-phosphatase activity, indicating that this compound is less potent than the compounds listed above. FLA 57 (representing half of the symmetrical structure of

Table 2 Comparison of the effects of disulfiram and its analogues on $[\text{H}]\text{Ins}(1,4,5)P_3$ dephosphorylation, TRH-stimulated phosphoinositide hydrolysis and $[\text{H}]\text{Ins}(1,4,5)P_3$ binding

For 5-phosphatase activity in soluble (Sol.) and particulate (Partic.) fractions from GH_3 cells, data are means $\pm$ S.D. ($n = 3-5$), using a 10 min preincubation period. The TRH-induced increase in inositol phosphate production in intact GH_3 cells in the presence of the test compound (following a preincubation period of 30 min) was compared with that in its absence and given as a percentage of the latter. Data are means $\pm$ S.D. ($n = 3$). For $[\text{H}]\text{Ins}(1,4,5)P_3$ binding to its receptor recognition site in bovine cerebellar membranes, the assay kit used did not allow for a preincubation phase. The values shown are means $\pm$ S.D. ($n = 3$).

Compound	Concn. (μM)	Activity (% of control values)				
		5-Phosphatase		TRH-stimulated inositol phosphate production		$\text{Ins}(1,4,5)P_3$ binding
		Sol.	Partic.	3 nM	100 nM	
Disulfiram	3	83 $\pm$ 1	79 $\pm$ 11			94 $\pm$ 9
	10	69 $\pm$ 5	69 $\pm$ 9			92 $\pm$ 9
	30	43 $\pm$ 2	43 $\pm$ 5	92 $\pm$ 17	85 $\pm$ 13	100 $\pm$ 7
	100	21 $\pm$ 5	15 $\pm$ 1	24 $\pm$ 20	19 $\pm$ 14	129 $\pm$ 21
	300	16 $\pm$ 11	20 $\pm$ 19	14 $\pm$ 15	3.2 $\pm$ 2	133 $\pm$ 20
FLA 46	3					102 $\pm$ 5
	10	66 $\pm$ 10	79 $\pm$ 6			98 $\pm$ 6
	20	59 $\pm$ 11	63 $\pm$ 3			
	30	38 $\pm$ 6	41 $\pm$ 6	106 $\pm$ 16	99 $\pm$ 11	98 $\pm$ 10
	100	25 $\pm$ 14	24 $\pm$ 18	48 $\pm$ 21	55 $\pm$ 24	136 $\pm$ 24
FLA 99	200	20 $\pm$ 7	22 $\pm$ 8			132 $\pm$ 30
	300			1.8 $\pm$ 3	6.8 $\pm$ 5	
	500	18 $\pm$ 8	15 $\pm$ 2			
	3	81 $\pm$ 3	78 $\pm$ 3			99 $\pm$ 6
	10	71 $\pm$ 14	66 $\pm$ 5			99 $\pm$ 2
EWP 815	30	67 $\pm$ 7	62 $\pm$ 11	144 $\pm$ 19	111 $\pm$ 8	97 $\pm$ 7
	100	30 $\pm$ 11	29 $\pm$ 5	113 $\pm$ 26	108 $\pm$ 16	131 $\pm$ 31
	300	12 $\pm$ 6	16 $\pm$ 7	138 $\pm$ 15	128 $\pm$ 11	142 $\pm$ 23
	3	84 $\pm$ 3	86 $\pm$ 3			106 $\pm$ 3
	10	46 $\pm$ 23	27 $\pm$ 13			98 $\pm$ 8
EWP 840	30	23 $\pm$ 8	19 $\pm$ 1	141 $\pm$ 42	101 $\pm$ 8	102 $\pm$ 1
	100	17 $\pm$ 3	13 $\pm$ 4	36 $\pm$ 5	30 $\pm$ 11	142 $\pm$ 13
	300	15 $\pm$ 3	8.8 $\pm$ 3	1.2 $\pm$ 2	1.8 $\pm$ 3	145 $\pm$ 10
	3	87 $\pm$ 3	80 $\pm$ 5			104 $\pm$ 3
	10	54 $\pm$ 25	40 $\pm$ 18			99 $\pm$ 8
	30	26 $\pm$ 12	20 $\pm$ 2	147 $\pm$ 18	134 $\pm$ 14	97 $\pm$ 13
	100	18 $\pm$ 4	17 $\pm$ 4	126 $\pm$ 17	110 $\pm$ 7	143 $\pm$ 10
	300	12 $\pm$ 2	11 $\pm$ 4	15 $\pm$ 10	15 $\pm$ 7	157 $\pm$ 12

Table 3 Effects of FLA 49, FLA 63, FLA 57, FLA 108 and L-cystine on GH₃ cell soluble and particulate 5-phosphatase activitiesData are either means $\pm$ S.D. ($n = 3$) or individual values, as appropriate, using a preincubation phase of 10 min.

Compound	Structure	Concn. (μ M)	5-Phosphatase activity (% of control)	
			Soluble	Particulate
FLA 49		100	67 $\pm$ 7	71 $\pm$ 11
FLA 63		1	96 $\pm$ 3	93 $\pm$ 4
		10	63 $\pm$ 18	38, 39
		100	28 $\pm$ 7	14, 18
		1000	17, 20	12, 12
FLA 57		100	92 $\pm$ 3	90 $\pm$ 4
FLA 108		100	97, 94	97, 91
L-Cystine		30	91 $\pm$ 7	103 $\pm$ 17
		100	91 $\pm$ 5	100 $\pm$ 11
		300	79 $\pm$ 4	92 $\pm$ 8

Figure 1 Inhibition of soluble (Δ , $\circ$, ∇) and particulate ($\blacktriangle$, $\bullet$, $\blacktriangledown$) 5-phosphatase activities from GH₃ cells by disulfiram (Δ , $\blacktriangle$), FLA 46 ($\circ$, $\bullet$) and 2,3-bisphosphoglycerate (∇ , $\blacktriangledown$)

Data are means $\pm$ S.D. ($n = 3-6$), using an [3 H]Ins(1,4,5) P_3 concentration of 10 μ M and a preincubation period of 10 min. Data for 2,3-bisphosphoglycerate, using the same assay and GH₃ cell preparations, were taken from Fowler and Brännström (1990).

FLA 63), the dithiocarbamic acid ethyl ester FLA 108 and L-cystine were all without effect on the 5-phosphatase activities at a concentration of 100 μ M (Table 3).

Heparin was found to inhibit the 5-phosphatase activities, with IC₅₀ values of 32 (soluble) and 79 (particulate) μ g/ml (results not shown).

The 5-phosphatase assay used can give information concerning inhibition of the Ins(1,4) P_2 phosphatase activity, which is localized in the soluble fraction (for review, see Shears, 1989). Thus, if a compound inhibits the Ins(1,4) P_2 phosphatase more potently than the Ins(1,4,5) P_3 5-phosphatase activity, the ratio of

3 H recovered as [3 H]Ins(1,4) P_2 /[3 H]Ins P_1 will be higher than expected in the soluble fraction. Such a pattern has been reported previously for glucose 6-phosphate (Fowler and Brännström, 1990), and was found in the present study for EWP 815 and EWP 840, but not for disulfiram, FLA 99 or heparin. Thus in one series of experiments, mean ($n = 3$) [3 H]Ins(1,4) P_2 /mean [3 H]Ins P recovery ratios of 0.6, 0.6, 0.7, 2.6 and 1.7 were found for control, disulfiram-, FLA 99-, EWP 815- and EWP 840-treated samples (all 30 μ M) respectively. In a second series of experiments using a different soluble preparation, values of 0.3, 0.5, 3.5/445, 2.3/21 and 0.2/0.3 were found for control samples and those treated with FLA 99 (300 μ M), EWP 815 (100/300 μ M), EWP 840 (100/300 μ M) and heparin (30 and 300 μ g/ml) respectively.

Mode of inhibition of Ins(1,4,5) P_3 5-phosphatase by FLA 99 and EWP 815

The time-dependence of the inhibition of soluble Ins(1,4,5) P_3 5-phosphatase activity by FLA 99 is shown in Figure 2. In the absence of preincubation, 50% inhibition was seen with 0.05 mM FLA 99, and the inhibition increased to > 90% as the preincubation time increased to 60 min. No such time-dependence, however, was seen with 0.005 mM FLA 99 (Figure 2). No significant variation in activity with preincubation time was found for the control samples preincubated with 2% DMSO alone (results not shown). Time courses of the inhibition were also determined in a single experiment using a particulate fraction. The percentage activities remaining after 0, 10, 20, 40 and 60 min of preincubation at 37 $^{\circ}$ C were: glucose 6-phosphate (25 mM), 20, 23, 19, 21 and 30%; FLA 99 (0.005 mM), 75, not determined, 44, 60 and 41%; FLA 99 (0.05 mM), 62, 29, 20, 7 and 4%. Thus the time-dependence of inhibition by 0.05 mM FLA 99 found in the soluble preparations was also observed when the particulate fraction was used.

Figure 2 Effect of 0.005 mM (○) and 0.05 mM (●) FLA 99 on the dephosphorylation of 10 μ M [3 H]Ins(1,4,5) P_3 by the soluble 5-phosphatase present in GH₃ cells

Data are means $\pm$ S.D. ($n = 3-4$) of the percentage activity remaining with respect to samples preincubated for the same length of time with 2% DMSO alone.

Figure 3 Lineweaver-Burk plot of the soluble 5-phosphatase activity in the absence (○) and presence of 0.01 (▲), 0.03 (▽), 0.1 (▼) and 0.3 (△) mM FLA 99

Incubation times of 5 min without any preincubation of enzyme and inhibitor were used. Data are means of three separate experiments.

The reversibility of the inhibition was assessed after a 60 min preincubation period using particulate fractions. Free inhibitor was removed by centrifugation of the membranes followed by resuspension in the same volume of assay buffer. This procedure effectively removed the inhibition produced by 50 mM glucose 6-phosphate, with values of $9 \pm 5\%$ and $91 \pm 6\%$ activity (means $\pm$ S.D., $n = 5$ and 3 respectively) for control and washed samples respectively. The corresponding values for 100 mM glucose 6-phosphate were $5 \pm 5\%$ ($n = 4$) and $78 \pm 1\%$ ($n = 3$) respectively. The values for the test compounds were: FLA 99 (0.005 mM), $48 \pm 6\%$ ($n = 5$) and $62 \pm 11\%$ ($n = 3$); FLA 99 (0.05 mM), $8 \pm 3\%$ ($n = 5$) and $11 \pm 1\%$ ($n = 3$); EWP 840 (0.01 mM), $24 \pm 14\%$ ($n = 5$) and $45 \pm 12\%$ ($n = 3$) respectively. The decrease in inhibition for both 0.005 mM FLA 99 and 0.01 mM EWP 840 borders on statistical significance

($0.1 > P > 0.05$, two-tailed t test), which may suggest the presence of both reversible and irreversible components. A Lineweaver-Burk plot of the inhibition produced by FLA 99 in the absence of preincubation is shown in Figure 3.

Effects of disulfiram and its analogues upon basal and TRH-stimulated phosphoinositide hydrolysis in intact GH₃ cells

In order to determine whether the production of Ins(1,4,5) P_3 in GH₃ cells is affected by disulfiram or its analogues, the rates of basal and TRH-stimulated phosphoinositide hydrolysis were determined. The assay used does not separate the [3 H]inositol phosphate products, so a relative increase, secondary to inhibition of the 5-phosphatase, in the recoveries of [3 H]Ins(1,4,5) P_3 and [3 H]Ins(1,3,4,5) P_4 at the expense of [3 H]Ins(1,3,4) P_3 and the bis- and mono-phosphates will not be seen. On the other hand, the effects of the compounds on the TRH receptors, the G-proteins coupling the TRH receptors to phosphoinositide-specific phospholipase C and phospholipase C itself will be observed.

Five compounds were tested: disulfiram, FLA 46, FLA 99, EWP 815 and EWP 840. None of these compounds affected basal phosphoinositide hydrolysis over the concentration range tested (30–300 μ M) (results not shown).

In the presence of 3 and 100 nM TRH, control phosphoinositide hydrolysis increased to values of 0.070 ± 0.019 and 0.157 ± 0.076 (3 H released as a fraction of 3 H incorporated; means $\pm$ S.D., $n = 3$) respectively, from a basal value of 0.033 ± 0.003 . This rate of increase is in line with the literature (see, e.g., Sharif et al., 1989). The effects of disulfiram, FLA 46, FLA 99, EWP 815 and EWP 840 on TRH-stimulated phosphoinositide hydrolysis are given in Table 2. FLA 99 did not inhibit the response to 3 or 100 nM TRH at any concentration tested (30, 100 and 300 μ M), whereas the other compounds were inhibitory. From these data, approximate IC_{50} values were calculated: disulfiram, 63 and 52 μ M; FLA 46, 89 and 110 μ M; EWP 815, 83 and 71 μ M; EWP 840, 220 and 200 μ M for 3 and 100 nM TRH respectively. The similarity of the EC_{50} values at the two TRH concentrations [corresponding to roughly 0.1 and 4 times the EC_{50} value for TRH (27 nM) reported by Sharif et al., 1989] would suggest that the compounds are not causing inhibition by substrate inactivation, since this would have the appearance of competitive inhibition.

Effects of disulfiram and its analogues on [3 H]Ins(1,4,5) P_3 binding to its receptor recognition site in bovine cerebellar membranes

The binding of [3 H]Ins(1,4,5) P_3 to its receptor was assayed at a ligand concentration of 1.2 nM using a NENQUEST kit. The specific binding was inhibited by non-radioactive Ins(1,4,5) P_3 and 2,3-bisphosphoglycerate, with IC_{50} values of 12 and 50 nM respectively (results not shown). Heparin inhibited binding with an IC_{50} value of 1.1 μ g/ml, and a concentration of 30 μ g/ml produced 97% inhibition of specific binding. These values are in line with the literature: thus, Varney et al. (1990) found a K_D value for Ins(1,4,5) P_3 of 9.8 nM and IC_{50} value of 3.8 μ g/ml for heparin using bovine cerebellum.

The effects of disulfiram, FLA 46, FLA 99, EWP 815 and EWP 840 upon the binding of [3 H]Ins(1,4,5) P_3 to its receptor recognition site in bovine cerebellar membranes were measured concomitantly with the effects of heparin, non-radioactive Ins(1,4,5) P_3 and 2,3-bisphosphoglycerate, and the data are shown in Table 2. None of the compounds inhibited the binding at any concentration tested (3–300 μ M), although a slight increase was observed at the highest concentrations tested of EWP 815, EWP 840 and, to a lesser extent, FLA 99.

Table 4 Effects of FLA 99 and EWP 840 on intracellular Ca^{2+} mobilization

The GH_3 cell suspensions were preincubated for 5 min with the test compounds prior to addition of TRH. Data are means $\pm$ S.D.

	$[\text{Ca}^{2+}]_i$ (nM)		<i>n</i>
	Basal	+ 30 nM TRH	
Control	110 $\pm$ 30	260 $\pm$ 40	9
+ 30 μM EWP 840	180 $\pm$ 17	264 $\pm$ 26	8
Control	176 $\pm$ 8	250 $\pm$ 12	4
+ 30 μM FLA 99	203 $\pm$ 12	240 $\pm$ 21	4

Table 5 *In vitro* toxicity of FLA 99 and EWP 840

Cells were plated out at a density of 0.33×10^6 cells/well in 6-well cluster plates. After 24 h the test compounds were added and the cells were cultured for a further 24 h prior to determination of cell densities. The percentage of control values refer to the increase in cell density (from 0.26×10^6 cells/well) during the 24 h incubation period with the test compound. Results are means of duplicate determinations for a single experiment.

Substance	Concentration (μM)	Cell density	
		($10^{-6} \times \text{no./well}$)	(% of control)
Control		0.55	100
0.5% DMSO		0.52	89
FLA 99	1	0.47	73
	3	0.48	75
	10	0.49	79
	30	0.53	92
	100	0.42	56
EWP 840	1	0.35	31
	3	0.068	< 0
	10	0.029	< 0

Effects of FLA 99 and EWP 840 on $[\text{Ca}^{2+}]_i$ in GH_3 cells

The effects of these two compounds on basal and 30 nM TRH-stimulated Ca^{2+} levels are shown in Table 4. Both compounds, at a concentration of 30 μM , increased the basal $[\text{Ca}^{2+}]_i$, but had no effect on the concentrations found in the presence of TRH. Higher concentrations of FLA 99 and EWP 840 greatly increased the $[\text{Ca}^{2+}]_i$ to $> 1 \mu\text{M}$ (results not shown).

In vitro toxicity of FLA 99 and EWP 840

GH_3 cells were incubated with FLA 99 or EWP 840 (300 μM) for 10, 20 and 60 min at 37 °C, after which time cell viability was determined by use of the Trypan Blue exclusion method. After these incubation times there was no obvious effect of the two compounds on the relative numbers of cells that were impermeable (viable) and permeable (non-viable) to Trypan Blue (results not shown). However, 100 μM FLA 99 reduced cell proliferation by 44% following a 24 h incubation (Table 5). EWP 840 was even more toxic in this test, with 1 μM decreasing cell proliferation by 69%, and concentrations of 3 and 10 μM totally blocking proliferation (Table 5).

DISCUSSION

In the present study, disulfiram and its analogues FLA 46, FLA 99, EWP 815 and EWP 840 have been assayed both for potency of inhibition of $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase activity and for effects on the production and function of $\text{Ins}(1,4,5)\text{P}_3$ in GH_3 cells. The compounds inhibit the soluble and particulate 5-phosphatase activities at concentrations in the micromolar range, making them (together with *p*-hydroxymercuribenzoate and *myo*-inositol 1,4,5-trisphosphorothiolate) among the most potent inhibitors of this enzyme so far reported (see, e.g., Downes et al., 1982; Erneux et al., 1986; Hansen et al., 1987; Cooke et al., 1989; Fowler and Brännström, 1990). Preliminary experiments also indicated that 1 mM oxidized glutathione inhibited the soluble 5-phosphatase activity by about 35%, whereas reduced glutathione at this concentration was without an inhibitory effect (C. J. Fowler and G. Brännström, unpublished work).

In the case of FLA 99, the inhibition was time-dependent. In the absence of preincubation, the compound was found to reduce the V_{max} value without changing the K_m value (Figure 3). In addition, the slope of the reciprocal plot reached a limit as the FLA 99 concentration increased (Figure 3). If it is assumed for the sake of argument that this component of the inhibition is reversible, as suggested by the decrease in inhibition upon washing of the samples, such a pattern of inhibition would be expected for a partial non-competitive inhibitor, where the substrate (S) and inhibitor (I) combine independently and reversibly to different enzyme (E) sites to produce ES, EI and ESI complexes, but where ESI can also produce product $[\text{Ins}(1,4)\text{P}_2]$, although not as efficiently as the ES complex (see Segal, 1975). However, it should be stressed that the evidence for a reversible component of the inhibition is not strong, and the data can be adequately explained by the suggestion that only one, or a defined small number, of thiol groups rapidly form mixed disulphides at low concentrations of test compounds, whereas higher concentrations react slowly with further groups. Whether different enzyme isoforms have differentially sensitive thiol groups awaits elucidation.

In addition to their effects on the 5-phosphatase, the effects of the compounds on other aspects of the phosphoinositide cycle were investigated. For all five compounds tested, the inhibition of 5-phosphatase activities was achieved at concentrations that did not affect the binding of $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ to its receptor recognition site (Table 2), although it should be mentioned as a caveat that the NENQUEST kit used for these experiments does not allow for a preincubation phase between inhibitor and receptor, and that the source of the $\text{Ins}(1,4,5)\text{P}_3$ recognition site was different, which may be of importance given the heterogeneity of this receptor (see Varney et al., 1990). Interestingly, higher concentrations of the compounds increased the observed binding, a finding in line with the report that thiol reagents increase the sensitivity of the $\text{Ins}(1,4,5)\text{P}_3$ receptor (Missiaen et al., 1991).

Effects of the compounds on $\text{Ins}(1,4,5)\text{P}_3$ receptor sensitivity and $\text{Ins}(1,4,5)\text{P}_3$ metabolism would be expected to have dramatic effects on the Ca^{2+} mobilization response to TRH stimulation in GH_3 cells, analogous to the effects of oxidized glutathione on hepatic cell Ca^{2+} signalling (Missiaen et al., 1991). A prerequisite for the study of such effects, however, is that the compounds in question do not affect the rate of production of $\text{Ins}(1,4,5)\text{P}_3$ following TRH receptor stimulation. This appears to be the case for disulfiram and FLA 46, but not for FLA 99 or, to a lesser extent, for EWP 840 (Table 2).

Both FLA 99 and EWP 840 produce large increases in the basal $[\text{Ca}^{2+}]_i$ of GH_3 cells (Table 4; see also the Results section). Such effects can be interpreted in three ways. (i) There may be a high basal metabolism of $\text{Ins}(1,4,5)\text{P}_3$ in these cells, so that its

disruption coupled with a sensitization of the Ins(1,4,5) P_3 receptor results in a dramatic mobilization of Ca^{2+} . In addition, effects of the compounds on the Ins(1,4,5) P_3 3-kinase and the Ins(1,3,4,5) P_4 receptor, which might also be expected to affect Ca^{2+} mobilization, cannot be ruled out at this stage. (ii) Other actions of the compound unrelated to the phosphoinositide cycle could lead to the increased Ca^{2+} concentration. (iii) The cell toxicity of the compounds may result in a permeabilization of the cell membrane to extracellular Ca^{2+} . Although the compounds did not increase the permeability of the cells to Trypan Blue, they were toxic, as demonstrated by their effects on cell proliferation (Table 5). Clearly this question cannot be resolved until selective (and less toxic) Ins(1,4,5) P_3 5-phosphatase inhibitors are developed.

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