Biochemical Pharmacology, Vol. 42, No. 5, pp. 1151–1153, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Ebselen prevents inositol (1,4,5)-trisphosphate binding to its receptor

(*Received* 9 *January* 1991; *accepted* 5 *May* 1991)

The anti-inflammatory effects of the seleno-organic compound ebselen (PZ51; 2-phenyl-1,2-benzoisoselenazol-3-(2*H*)-one) are established [1]. *In vitro*, the compound catalyses a glutathione peroxidase-like reaction [2] and thereby is supposed to lower the peroxide tonus required for lipoxygenase and cyclooxygenase activities [2]. Recently we demonstrated that ebselen inhibits aggregation of aspirin-treated human platelets [3] which could be related to an altered calcium homeostasis. We anticipated an inhibitory effect of ebselen on the inositol 1,4,5-trisphosphate (IP_3)-induced calcium release from experiments with intact platelets as well as isolated platelet microsomal vesicles.

Evidently sulfhydryl groups are important for the IP_3-induced calcium release from the dense tubular system of human platelets [4]. It has also been reported by Guillemette and Sequi [5] and Supattapone *et al.* [6] that the IP_3 receptor contains disulfide bridges as well as free sulfhydryl groups which are both essential for the IP_3 binding and the calcium release. Since ebselen reacts with endogenous thiol groups in proteins [7–9], we suggest, on the basis of the experiments reported here, that ebselen affects the IP_3-induced calcium release from the dense tubular systems by inhibiting IP_3 binding to its specific receptor.

Materials and Methods

Ebselen and its derivative 2-methylselenobenzanilide were a gift from Dr E. Graf (Rhône-Poulenc/Nattermann Co., Cologne Research Center, F.R.G.) Stock solutions were made up in dimethylsulfoxide. [^{3}H]Inositol 1,4,5-trisphosphate ([^{3}H]IP_3) (56.5 Ci/mmol) was obtained from Amersham International (Amersham, U.K.). Fura-2 (free acid) was delivered by Boehringer (Mannheim, F.R.G.) and the SH reagents were purchased from Sigma Chemie (Deisenhofen, F.R.G.). Other chemicals were of the highest grade of purity available and came from local commercial sources.

Preparation of platelet rich-plasma and microsomes. Preparation of platelet rich-plasma and microsomes were carried out mainly as described previously [3].

Calcium measurements. Characterizing calcium movements in platelet microsomes we followed the protocol of Enouf *et al.* [10] except that the ATP concentration in the homogenizing buffer as well as in the incubation buffer was reduced to 2.5 mM. Calcium signals were recorded after adding 3 μM fura-2 (free acid) to the platelet microsomal fraction (0.5 mg/mL) at 37°. The fluorescence signal above 450 nm was monitored after excitation at 335 and 380 nm, respectively.

[^{3}H]*Inositol 1,4,5-trisphosphate binding.* [^{3}H]IP_3 binding was determined essentially as previously outlined by Varney *et al.* [11]. All incubations (10 min, 4°) contained 0.2 mg protein and 1 nM [^{3}H]IP_3 (22.000 ndpm/assay). Non-specific binding was determined after the addition of 10 μM unlabelled IP_3. The assays containing displacing agents were preincubated for 15 min at 37°.

Results and Discussion

In the present study we investigated the modulation of microsomal calcium release and IP_3 binding to its receptor by ebselen. In order to prove the reliability of a functional IP_3 compartment we characterized the IP_3-receptor by the affinity towards heparin. Varney *et al.* [11] determined the heparin displacement of IP_3 using various tissues (IC_{50} for platelet microsomes: 4.1 μg/mL). We measured 27% inhibition of the specific IP_3 binding in the presence of 3 μg/mL heparin. This illustrates that our microsomal system gives comparable results to literature data and contains the otherwise characterized IP_3-binding protein.

Inhibition of the IP_3- (3 μM) induced Ca^{2+} release from human platelet microsomes by ebselen is shown in Fig. 1. Half maximal inhibition was observed at 1.0 ± 0.2 μM ebselen (mean ± SD, N = 4). At this concentration the Ca^{2+} sequestration system was not affected as judged from the original fura-2 recordings. When microsomal vesicles were loaded with 10 μM $CaCl_2$ in the presence of 2.5 mM ATP, added IP_3 (3 μM) released 20–30% (average value from five different determinations) of the total ionomycin (100 μM) releasable Ca^{2+} content of the microsomes.

To further characterize the inhibitory effect of ebselen we investigated the [^{3}H]IP_3 binding to human platelet membranes. As shown in Fig. 2, ebselen displaced the specific IP_3 binding with an IC_{50} value of 5.0 ± 1.2 μM (mean ± SD, N = 4). This value is somewhat higher than the IC_{50} for the calcium release reaction but one should consider the higher protein content, the different temperature and cofactor additions or the different pH values as possible causes for these differences. Especially, comparing the preparation of the protein for the two assays [10, 11] and taking into account the covalent binding of ebselen to proteins one should expect higher IC_{50} values in the binding assay. All the individual factors mentioned affect IP_3 binding and IP_3-induced calcium release in different ways [5, 11–13] and so do not allow identical assay conditions in both cases.

The ebselen derivative 2-methylselenobenzanilide showed no efficiency under any of the conditions tested (data not shown). This points to the importance of a free available seleno group and makes unspecific effects of ebselen unlikely. SH-reducing agents like dithiothreitol (DTT) showed a strong preventing effect on ebselen-

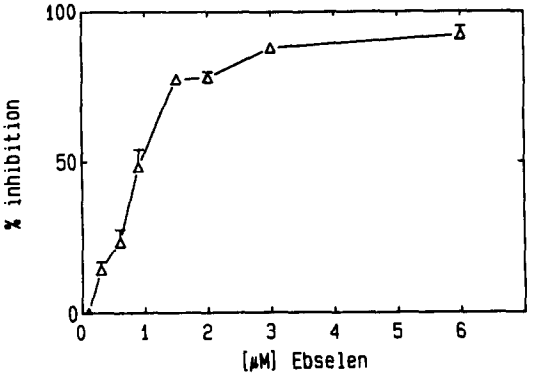

Fig. 1. Ebselen inhibits the IP_3-induced Ca^{2+} release. Assay conditions were used as described in Materials and Methods using human platelet microsomes (0.5 mg protein/assay). Different concentrations of ebselen were incubated for 3 min prior to addition of 3 μM IP_3 to release stored calcium. Values are mean ± SD from four different preparations.

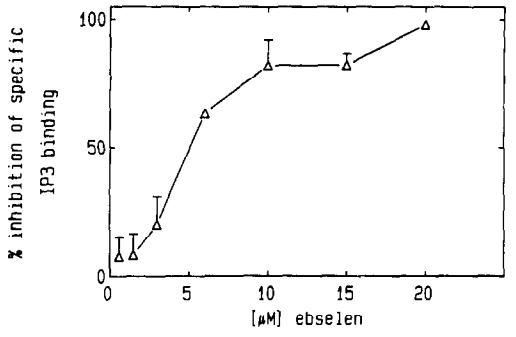

Fig. 2. Inhibition of the [^{3}H]IP$_3$ binding by ebselen. [^{3}H][IP$_3$ binding to human platelet membranes was determined as outlined in Materials and Methods. All assays containing ebselen were preincubated for 15 min at 37°. Results are mean ± SD from four different preparations.

modulated IP$_3$ binding as well as on IP$_3$-induced calcium release. Preincubation with 1 mM DTT at 37° for 3 min almost completely prevented the inhibition of the IP$_3$ induced calcium release caused by 2 μM ebselen to 6.0 ± 14% (mean ± SD, N = 3). In control incubations (without DTT) 2 μM ebselen caused more than 80% inhibition of the IP$_3$-induced calcium release (see Fig. 1). In analogy the inhibitory effect of 10 μM ebselen on the [^{3}H]IP$_3$ binding was reduced from about 80% (see Fig. 2) to 20 ± 9.6% (mean ± SD, N = 3) in the presence of DTT. DTT seems to be equally effective in both situations when either ebselen was incubated first and DTT was added latter or when DTT was preincubated and ebselen was introduced afterwards. Therefore DTT not only prevents but also reverses the ebselen effect, most likely by cleaving a covalent binding between a protein and ebselen. A reaction of ebselen with thiols is more than likely [7] but this possibility cannot explain the reversible effect of DTT in our system. DTT protects SH groups against oxidation and as shown also eliminates the ebselen effect, showing no interference with the parameters determined. Therefore, we conclude an interference of ebselen with critical sulfhydryl groups near the IP$_3$ binding site of platelet membrane vesicles. This assumption was strengthened by showing the binding of [^{14}C]ebselen to several distinct microsomal proteins (data not shown) and its reversibility by DTT. A further characterization of the labelled proteins is necessary to definitely point out the IP$_3$ receptor.

For comparison we tested several other SH reagents evaluating their efficiency on the [^{3}H]IP$_3$ binding to the platelet calcium pool. As known from the literature the alkylating agent *N*-methylamaleimide (NEM) and the SH-reactive compound *p*-chloromercurobenzoyl sulfonate (*p*-CMBS) affect the IP$_3$ binding to rat liver plasma membrane [12] as well as to rat cerebellum membranes [6]. The data given in Table 1 revealed that compared to ebselen quite high concentrations of NEM and *p*-CMBS are necessary to cause a fairly complete inhibition of the specific IP$_3$ binding using platelet membranes. Despite the higher concentrations necessary this information supports our assumption that in the case of ebselen also a modulation of SH groups is a likely mechanism. Using the calcium release assay we were looking for the combined effect of ebselen and NEM. Addition of ebselen and NEM alone, below maximal effective concentrations, and both compounds together revealed a clearly additive effect. Ebselen (0.6 μM) and NEM (200 μM) showed 19 ± 8% and 43 ± 4% inhibition of the IP$_3$-induced calcium release, respectively (mean ± SD, N = 3). The combination of ebselen and NEM (same concentrations) resulted in an additive effect, showing around 60% inhibition.

Table 1. SH reagents affect [^{3}H]IP$_3$ binding

Compound	% Inhibition of specific [^{3}H]IP$_3$ binding
2,2'DTDP (6 μM)	39.0 ± 6.9
4,4'DTDP (6 μM)	36.3 ± 16.3
NEM (200 μM)	70.1 ± 8.5
p-CMBS (1 mM)	90.0 ± 10.5

Assay conditions are as in Fig. 2. Data refer to incubations containing the solvent (DMSO) as vehicle control (0% inhibition).
Values are means ± SD from three different preparations.

"Reactive" disulfides are known to specifically oxidize free SH sites via a thiol–disulfide exchange reaction and to trigger calcium release from sarcoplasmic reticulum vesicles [14]. The compound 2,2'-dithiodipyridine (2,2'DTDP) (preincubated for 3 min) at a concentration of 6 μM showed 77 ± 6.5% inhibition (mean ± SD, N = 3) of the IP$_3$-induced calcium release, whereas the binding of IP$_3$ was inhibited by about 40% (Table 1). Similar results were obtained using 4,4'-dithiodipyridine (4,4'DTDP), another compound belonging to the class of "reactive" disulfides. The chosen concentrations of 2,2'DTDP and 4,4'DTDP do not release calcium from platelet vesicles although they do so at higher concentrations.

The available data support our hypothesis that ebselen interferes with sulfhydryl groups at or near the IP$_3$ binding site and causes an inhibition of the calcium release. Our evidence is:

1. Reducing agents like DTT diminish the inhibitory effect of ebselen on IP$_3$-induced calcium release and IP$_3$ binding.
2. SH-reactive agents like NEM and *p*-CMBS affect the IP$_3$ binding in a similar way.
3. An analogous interference of calcium movements and IP$_3$ binding is caused by reactive disulfides (2,2'DTDP and 4,4'DTDP; in a similar concentration range compared to ebselen) which are known to oxidize critical sulfhydryl groups catalysing a thiol–disulfide exchange reaction.
4. Ebselen reacts with endogenous thiols to form selenyl sulfides [7] as well as with protein thiol groups [8, 9].

Our data suggest that ebselen binds to sulfhydryl groups linked to the IP$_3$-sensitive calcium pool. This modification inhibits the IP$_3$ binding and concomitantly the IP$_3$-induced calcium release. These experiments support our previous observation that ebselen inhibits platelet activation by modulating intracellular calcium homeostasis suggesting the possibility that the same mechanism is valid for whole cell experiments. Inhibition of calcium release from the inositol 1,4,5-trisphosphate sensitive pool in granulocytes and macrophages may contribute to the anti-inflammatory properties of this compound although the *in-vivo* relevance remains to be established.

Acknowledgements—The study was supported by the Deutsche Forschungsgemeinschaft (SFB 156/A4).

* To whom correspondence should be addressed: University of Konstanz, Faculty of Biology, Universitätsstr. 8–10, Postfach 5560, 7750 Konstanz, F.R.G.

Faculty of Biology
University of Konstanz
Federal Republic of Germany

STEFANIE DIMMELER
BERNHARD BRÜNE*
VOLKER ULLRICH

REFERENCES

1. Parnham MJ and Graf E, Seleno-organic compounds and the therapy of hydroperoxide-linked pathological conditions. *Biochem Pharmacol* **36**: 3095–3102, 1987.
2. Müller A, Candenas E, Graf P and Sies H, A novel biologically active seleno-organic compound-I. Glutathion peroxidase-like activity *in vitro* and antioxidant capacity of PZ 51 (ebselen). *Biochem Pharmacol* **33**: 3235–3239, 1984.
3. Brüne B, Diewald B and Ullrich V, Ebselen affects calcium homeostatis in human platelets. *Biochem Pharmacol* **41**: 1805–1811, 1991.
4. Adunyah SE and Dean WL, Effects of sulhydryl reagents and other inhibitors on Ca^{2+} transport and inositol trisphosphate-induced Ca^{2+} release from human platelets membranes. *J Biol Chem* **261**: 13071–13075, 1986.
5. Guillemette G and Sequi JA, Effects of pH, reducing and alkylating reagents on the binding and Ca^{2+} release activities of inositol 1,4,5-trisphosphate in bovine adrenal cortex. *Mol Endocrinol* **2**: 1249–1525, 1988.
6. Supattapone S, Worley PF, Baraban JM and Snyder SH, Solubilization, purification, and characterization of an inositol trisphosphate receptor. *J Biol Chem* **263**: 1530–1534, 1988.
7. Haenen GRMM, De Rooij BM, Vermeulen NPE and Bast A, Mechanism of the reaction of ebselen with endogenous thiols: dihydrolipoate is a better cofactor than glutathion in the peroxidase activity of ebselen. *Mol Pharmacol* **37**: 412–422, 1989.
8. Cotgreave IA, Duddy SK, Kass GEN, Thompson D and Moldéus P, Studies on the anti-inflammatory activity of ebselen. Ebselen interferes with granulocyte oxidative burst by dual inhibition of NADPH oxidase and protein kinase C. *Biochem Pharmacol* **38**: 649–656, 1989.
9. Kühn-Velten N and Sies H, Optical spectral studies of ebselen interaction with cytochrome P-450 of rat liver microsomes. *Biochem Pharmacol* **38**: 619–625, 1989.
10. Enouf J, Bredoux R and Levy-Toledano S, Characterization of calcium liberation from human platelet membrane fraction. *Biochim Biophys Acta* **772**: 251–258, 1984.
11. Varney MA, Rivera J, Lopez Bernal A and Watson SP, Are there subtypes of the inositol 1,4,5-trisphosphate receptor? *Biochem J* **269**: 211–216, 1990.
12. Pruijn FB, Sibeijn J-P and Bast A, Changes in inositol 1,4,5-trisphosphate binding to hepatic plasma membranes caused by temperature, *N*-ethylmaleimide and menadione. *Biochem Pharmacol* **40**: 1947–1952, 1990.
13. Worley PF, Baraban JM, Supattapone S, Wilson VS and Snyder SH, Characterization of inositol trisphosphate receptor binding in brain. *J Biol Chem* **262**: 12132–12136, 1987.
14. Zaidi NF, Langenaur CF, Abramson JJ, Pessah I and Salama G, Reactive disulfides trigger Ca^2 release from sarcoplasmic reticulum via an oxidation reaction. *J Biol Chem* **36**: 21725–21736, 1989.