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EBSELEN AFFECTS CALCIUM HOMEOSTASIS IN HUMAN PLATELETS

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Abstract—Ebselen (PZ 51, 2-phenyl-1,2-benzoisoselenazol-3-(2H)-one) is a selenoorganic compound with anti-inflammatory properties. Its pharmacological action is thought to originate from its peroxidase activity which could lower the peroxide tonus required for cyclooxygenase and lipoxygenase activations. From experiments with aspirin-treated human platelets we now present evidence that ebselen also affects intracellular calcium homeostasis by inhibiting the agonist-triggered increase in intracellular calcium. Using Mn^{2+} entry to quench the fura-2 fluorescence after cell stimulation, we could exclude an interaction of ebselen with receptor-operated calcium channels and therefore an inhibition of extracellular calcium influx. It became evident from whole cell experiments and by using isolated platelet microsomal vesicles that ebselen inhibits the inositol 1,4,5-trisphosphate (IP_3) induced calcium release. Besides this inhibitory effect of ebselen on the calcium release higher concentrations of the compound ($\geq 5\ \mu M$) induced a calcium release from our microsomal vesicles which also could be reversed by dithiothreitol. An activation of inflammatory cells is usually associated with increased cytosolic calcium concentrations. An inhibition of such calcium movements by ebselen may account for an up to now unidentified anti-inflammatory mechanism of ebselen action which is linked to a direct effect of this compound rather than to its peroxidase-like activity.

Ebselen (PZ 51, 2-phenyl-1,2-benzoisoselenazol-3-(2H)-one) is an organoselenium heterocycle which has been shown to be an efficient anti-inflammatory compound. The protective activity was demonstrated using several model inflammatory conditions in animals, including cobra venom paw edema [1], monoarthritis [2], alveolitis and broncheolitis [3]. Ebselen also protected against endotoxin shock in mice sensitised by galactosamine [4] and shows several pharmacodynamic activities employing various *in vitro* systems. It has been reported that ebselen exhibits GSH peroxidase-like activity [5–7], inhibits ADP-Fe induced lipid peroxidation [8] and decreases diquat-induced cytotoxicity in isolated hepatocytes [9]. Ebselen can act as a potent antioxidant inhibiting lipid peroxidation in microsomes and isolated cells [5, 8, 9], it inhibits human granulocyte oxidative burst and was shown to inhibit protein kinase C [10]. The reported glutathione peroxidase-like activity of ebselen was demonstrated to be involved in the conversion of LTB_4 to its biologically inactive 6-trans isomer [11]. In addition, ebselen directly inhibits mammalian leukocyte 5-lipoxygenase [12] and platelet 12-lipoxygenase as well as platelet cyclooxygenase [13]. At present, the underlying mechanism of the anti-inflammatory activity of ebselen is still not fully elucidated and it is uncertain which of the numerous activities is responsible for the efficiency in the different *in vivo* and *in vitro* systems. Here we present observations on the effect of ebselen on platelet functions different from the well documented classical non-steroidal anti-inflammatory behaviour of ebselen. The results document a further pharmacodynamic property of ebselen in relation to calcium movements in aspirin-treated human platelets. On the one hand we can establish an inhibitory activity of low ebselen concentrations on the inositol 1,4,5-trisphosphate induced calcium release from platelet microsomes, whereas on the other hand we are able to demonstrate a calcium release property at high ebselen concentrations.

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MATERIALS AND METHODS

Chemicals

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 (DMSO). Prostacyclin was kindly provided from Schering (Berlin, F.R.G.). Radiochemicals were bought from Du Pont (Dreieich, F.R.G.). Fura-2-acetoxymethyl and inositol 1,4,5-trisphosphate were delivered by Calbiochem (Frankfurt a.M., F.R.G.), and thrombin was purchased from Hoffmann-La Roche (Basel, Switzerland). All other reagents were of the highest grade of purity available and were obtained from local commercial sources.

Methods

Preparation of platelet rich plasma and washed human platelets. Preparation of platelet rich plasma and suspensions of washed human platelets were mainly carried out as previously outlined [14]. In order to inhibit platelet cyclooxygenase, platelet rich plasma was incubated with 1 mM aspirin (dissolved in ethanol) for 30 min before starting the washing procedure. Complete inhibition of platelet

cyclooxygenase was assured by the inability of arachidonic acid to induce platelet activation. Aggregation studies were performed according to the same reference cited.

Preparation of ^{32}P-*labeled platelets.* In this case we followed the method described by Crouch *et al.* [15]. Gels were subjected to autoradiography, the bands were cut out and counted in a liquid scintillation counter.

Calcium measurements. Calcium measurements in intact platelets were carried out as stated in Ref. 14. External calcium was adjusted by the addition of 1 mM $CaCl_2$ or by adding ethyleneglycolbis-(aminoethylether)tetra-acetate (EGTA) to give a final free extracellular calcium concentration.

Characterization of calcium movements in a vesicular platelet membrane fraction was carried out as described by Enouf *et al.* [16] except that for the preparation of the membrane fraction the following modifications were made: the ATP concentration was reduced to 2.5 mM in the homogenizing as well as in the incubation buffer and potassium oxalate was replaced by potassium phosphate. Calcium movements were recorded after adding 3 μM fura-2 (free acid) to the platelet microsomal fraction (0.5 mg/mL). The fluorescence signal above 450 nm was monitored after excitation at 335 and 380 nm, respectively. Calcium release was calculated as percentage of the maximal release brought about by 10 μM ionomycin. In order to remove ATP from the incubation medium we added 5 mM glucose and hexokinase (3 μg/mL).

Calcium influx into platelets was measured using the manganese technique described by Sage *et al.* [17]. This technique has previously been employed to show that stimulation of receptor mediated calcium entry will result in the concomitant stimulation of Mn^{2+} influx and is based on the observation that Mn^{2+} binds to fura-2 and quenches its fluorescence. The use of the two excitation wavelengths 335 and 364 nm respectively allows monitoring at a calcium-sensitive and a relatively calcium-insensitive wavelength at the same time. The use of excitation at 364 nm thus allows the selective study of Mn^{2+} entry without interference of the signal caused by changes in $[Ca^{2+}]_i$.† Using the manganese quenching technique or isolated microsomal preparations in order to study calcium movements (see Figs 4–6 later) only representative traces of at least five similar experiments (different platelet or microsomal preparations) are shown.

Measurements of the "PI-response". The agonist-stimulated activation of platelet phospholipase C was determined employing two different assay systems: The inositol 1,4,5-trisphosphate generation was determined with a competitive protein binding assay from Amersham Corp. Reactions with 5×10^8 platelets/mL were stopped after 30 sec and the assay was carried out as outlined in the company manual. The assay was validated by the use of a high specific activity tritiated IP_3 tracer together with a specific and sensitive IP_3 binding protein. Results were calculated by the use of an IP_3-standard curve. Besides looking for the generation of IP_3 we also determined phosphatidic acid from ^{14}C-labeled platelets as a relative marker of phospholipase C activation by a method described in Ref. 14. Aliquots (0.5 mL) of ^{14}C-labeled cells were transferred into tubes containing 1.4 mL of chloroform/methanol 1:2 (v/v) after the addition of 0.5 mL of methanol in order to stop the reaction. Samples were partitioned by adding 0.6 mL chloroform and 0.6 mL of formic acid in order to reach pH 3 for lipid extraction.

Phosphatidic acid was separated by thin layer chromatography as described. ^{14}C-Radioactivity of phosphatidic acid in control samples was about 200 cpm.

† Abbreviations: DTS, dense tubular system; IP_3, inositol 1,4,5-trisphosphate; DTT, dithiothreitol; $[Ca^{2+}]_i$, intracellular calcium; PI-response, phosphatidylinositol 4,5-bisphosphate hydrolysis.

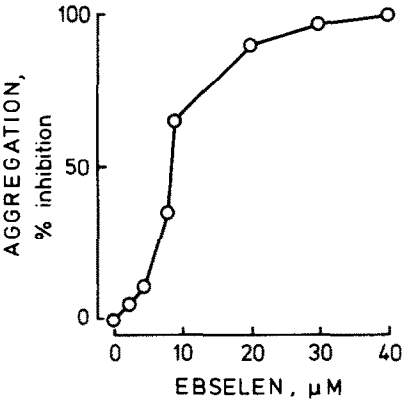

Fig. 1. The effect of ebselen on the aggregation of aspirin-treated human platelets. Aspirin-treated washed human platelets were incubated for 2 min with the indicated concentrations of ebselen before stimulation with 0.1 units/mL thrombin. Inhibition is calculated compared to control values without ebselen. Preparation of platelet suspensions and aggregation studies are carried out as given under Materials and Methods.

RESULTS

We have first studied the effects of ebselen and the ebselen derivative 2-methylselenobenzanilide on the aggregation of aspirin-treated washed human platelets. Ebselen inhibits the aggregatory response after stimulation with 0.1 units/mL thrombin dose-dependently as shown in Fig. 1.

From the sigmoidal dose–response curve an IC_{50} for the inhibition after challenging with thrombin was estimated as 9 ± 2 μM (mean ± SD from four different experiments). After adding the calcium ionophore A23187 or the prostaglandin endoperoxide analogue U46619 just above threshold concentrations in order to induce aggregation, ebselen showed a similar inhibitory activity at concentrations around 10 μM. The methyl derivative of ebselen, 2-methylselenobenzanilide, was inactive in any of the conditions tested. Performing the same set of experiments in platelets which had not been treated with aspirin, the IC_{50} for the inhibition of aggregation

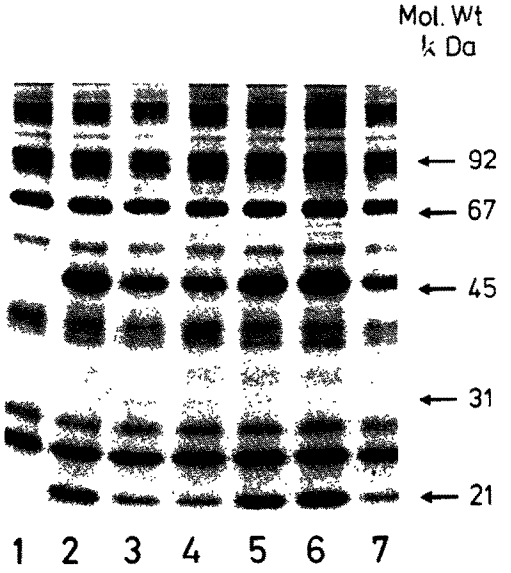

Fig. 2. Proteinphosphorylation of cytosolic platelet proteins and its modulation by ebselen and 2-methylselenobenzanilide. Aspirin-treated platelets were incubated with [^{32}P]P_i for 90 min, washed and resuspended in buffer without label. Samples were then challenged with thrombin (0.1 units/mL) alone or in combination with ebselen or its analogue (preincubated for 2 min). After 20 sec (lanes 1–5) or 40 sec (lanes 6 and 7) aliquots were taken, the reaction terminated, and proteins separated by SDS–PAGE. Radioactive bands were localized by autoradiography which is shown in the figure. For other details see Materials and Methods. Lanes: (1) control, incubation without addition; (2) thrombin; (3) 50 μM ebselen plus thrombin; (4) 100 μM ebselen plus thrombin; (5) 100 μM 2-methylselenobenzanilide plus thrombin; (6) thrombin; (7) 100 μM ebselen plus thrombin.

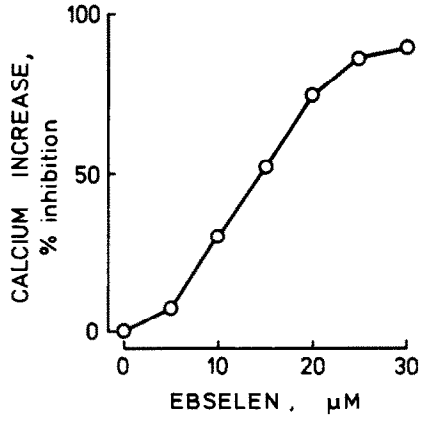

Fig. 3. Inhibition of a cytosolic calcium increase by ebselen in fura-2 loaded platelets. Measurement and calculation of cytosolic free calcium was carried out as detailed in Materials and Methods. Inhibition is calculated compared to control values obtained in the absence of ebselen. Experiments were carried out in the presence of 1 mM EDTA and 0.1 units/mL thrombin as an agonist.

by ebselen after stimulation with 4 μM arachidonic acid was 2 ± 1 μM (mean ± SD, N = 3). This inhibitory property of ebselen is well known and could be traced back to platelet cyclooxygenase inhibition, but since the previous and all further experiments were carried out in aspirin-treated platelets, these results clearly demonstrate that there is an additional interference of ebselen with the cascade of platelet aggregation. In order to gain further insight into this action of ebselen we investigated its effect on the protein phosphorylation pattern, since an inhibition of the calcium/phospholipid-dependent protein kinase C had already been reported [10]. Although such experiments had been performed on soluble and enriched protein kinase C preparations, we expected similar results in whole platelets. When aspirin-treated platelets were labeled with [^{32}P]P_i the pattern of protein phosphorylation in the presence and absence of ebselen after stimulation with 0.1 units/mL thrombin appears as shown in the representative results of Fig. 2.

Autoradiography of an 11% SDS–gel revealed that thrombin (lane 2) induces a rapid (20 sec incubation) phosphorylation of the classical platelet protein kinase C substrate, the 47 kDa protein, as well as the phosphorylation of a 20 kDa protein, which very likely is myosin-light chain. Addition of ebselen in concentrations of 50 and 100 μM, respectively (lanes 3 and 4) 2 min before the agonist resulted in a marked decrease in phosphorylation of both, the 47 kDa as well as the 20 kDa proteins. Counting the radioactivity of the 47 kDa bands revealed that 50 μM ebselen inhibited the phosphorylation about 40%, while 100 μM ebselen brought about 60% inhibition compared to controls. Replacing ebselen by 100 μM of 2-methylselenobenzanilide (lane 5) resulted in no significant inhibitory effect. Lane 6 shows the phosphorylation pattern after the addition of 0.1 units/mL thrombin and an incubation period of 40 sec. Again a concentration of 100 μM ebselen (lane 7) inhibited the phosphorylation of the 47 kDa as well as of the 20 kDa protein. These results confirm an inhibitory activity of ebselen on the activity of platelet protein kinase C. However, the effective concentrations were relatively high. We therefore conclude that this inhibition of protein kinase C may not be related to the observed inhibitory effect during platelet activation. It is also interesting that the phosphorylation of the 20 kDa protein is affected as well. Therefore the effect does not seem to be specific for protein kinase C, and possibly involves different protein kinases as well.

A critical parameter for platelet aggregation is the mobilization of intracellular calcium which could have been affected by ebselen. In aspirin-treated platelets ebselen, in a concentration-dependent fashion, inhibits the cytosolic calcium increase in fura-2 loaded thrombocytes, after stimulation with 0.1 units/mL thrombin (Fig. 3).

The IC_{50} value under these conditions was 14 ± 4 μM (mean ± SD, N = 3). These experiments were carried out in the presence of 1 mM extracellular EDTA. Therefore the inhibition should represent a blocking effect on the calcium release from the dense tubular system, which comprises the IP_3-releasable

calcium store. It is important to mention that while doing calcium measurements in whole cells in the presence of 1 mM extracellular calcium higher concentrations of ebselen (ebselen $\geq 25\ \mu M$) caused an immediate calcium influx.

In order to elucidate whether the observed inhibitory effect on the calcium mobilization may result from an impaired PI-response we determined the effect of ebselen on the formation of phosphatidic acid and inositol 1,4,5-trisphosphate. The formation of phosphatidic acid in platelets is closely correlated with the activation of phospholipase C [18]. Performing three sets of experiments with triplicate determinations and different concentrations of ebselen, the formation of phosphatidic acid was calculated to be $100 \pm 28\%$ (mean $\pm$ SD, $N = 3$) compared to controls. Because the conclusions from these results are very critical, we also measured the activity of phospholipase C by quantifying the amount of generated IP_3 with a protein binding assay (single determination). Aspirin-treated platelets were stimulated with 0.1 units/mL thrombin for 30 sec in the presence of 20 μM ebselen or in a second experiment with 1 μg/mL prostacyclin. Thrombin elevated the IP_3 content 320% above control values (control incubations contained 0.41 pmol IP_3/assay; set as 100% control value) and this increase was completely inhibited by the addition of prostacyclin. In contrast, ebselen does not inhibit the thrombin-induced IP_3 formation at all. Given alone it raised the IP_3 content to about 400% above control values and the combination of ebselen and thrombin showed about 600% elevated IP_3 levels. Recording the aggregatory response during this initial phase of aggregation showed a complete inhibition of the thrombin induced aggregation by prostacyclin as well as by ebselen. In the ebselen-treated samples we observed a shape change but no aggregation. Both assay systems indicate that the activity of phospholipase C is unaffected, and therefore, despite blocking of the aggregation IP_3, as well as 1,2-diacylglycerol, is being formed.

From these observations we concluded that the main inhibitory activity of ebselen was on calcium mobilization and/or redistribution of calcium from the dense tubular system or the whole cell. Therefore we investigated the effect of ebselen on the calcium influx by monitoring divalent cation entry into fura-2 loaded cells in the presence of 500 μM extracellular Mn^{2+}. Figure 4 shows the effect of 20 μM ebselen on the fluorescence quenching properties of Mn^{2+} after the addition of 0.1 units/mL thrombin.

Monitoring the fluorescence at the calcium-sensitive wavelength (335 nm) indicated a fluorescence increase after the addition of thrombin, followed by subsequent fluorescence quenching due to Mn^{2+} influx. Ebselen only reduced the transient cytosolic calcium increase induced by the platelet agonist. Looking for the fura-2 fluorescence at the calcium insensitive but still Mn^{2+} sensitive wavelength (364 nm) revealed that ebselen did not alter the rate of fura-2 fluorescence quenching. The addition of 10 μM ionomycin showed no substantial further effect on the fluorescence quenching. In all experiments the Mn^{2+} quenching effects could be blocked by the addition of 2 mM external Ni^{2+} (data

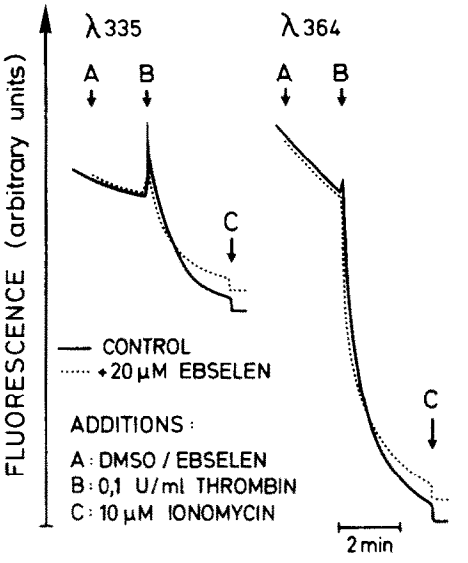

Fig. 4. Effect of Mn^{2+} on fura-2 fluorescence of platelets stimulated with thrombin in the presence and absence of ebselen. Fura-2 fluorescence was monitored as described in Materials and Methods at the calcium sensitive wavelength 335 nm and the calcium insensitive wavelength 364 nm. Ebselen (20 μM) and 0.1 units/mL thrombin were added at the times indicated.

not shown). Therefore, the opening of receptor-operated Ca^{2+} channels is not influenced by ebselen. It should be mentioned that addition of higher concentrations of ebselen alone (ebselen $\geq 25\ \mu M$) resulted in an immediate partial fura-2 quenching. This is probably related to an ebselen-mediated Mn^{2+} influx through divalent cation channels because manganese entry could be modified by the addition of nickel. Subsequent addition of an agonist like thrombin resulted in a further fura-2 quenching, indicating that these ebselen concentrations neither affect cell integrity nor influence the normal signal transduction pathways.

In additional experiments we studied the effect of ebselen on calcium uptake and efflux in microsomal vesicles. Our microsomal preparations in the presence of ATP sequestered 10 μM added calcium completely. Inositol 1,4,5-trisphosphate (3 μM) released between 20 and 30% of the stored calcium (an average value originating from more than 10 different experiments), calculated as percentage of the maximal ionomycin releasable calcium. Figure 5A shows that in the presence of ATP calcium is rapidly taken up into the vesicles and that IP_3 releases about 20% of the stored calcium which is sequestered shortly after being released. Adding ionomycin released all calcium from the microsomal vesicles. A concentration of 2.5 μM ebselen added 2 min before the IP_3 stimulus reduces the IP_3 induced calcium release nearly completely.

Figure 5B illustrates that a concentration of 2.5 μM ebselen added before $CaCl_2$ is introduced does not affect the calcium uptake system of our microsomal preparation. Therefore, an inhibition of the ATP driven Ca^{2+} pump at a concentration of 2.5 μM

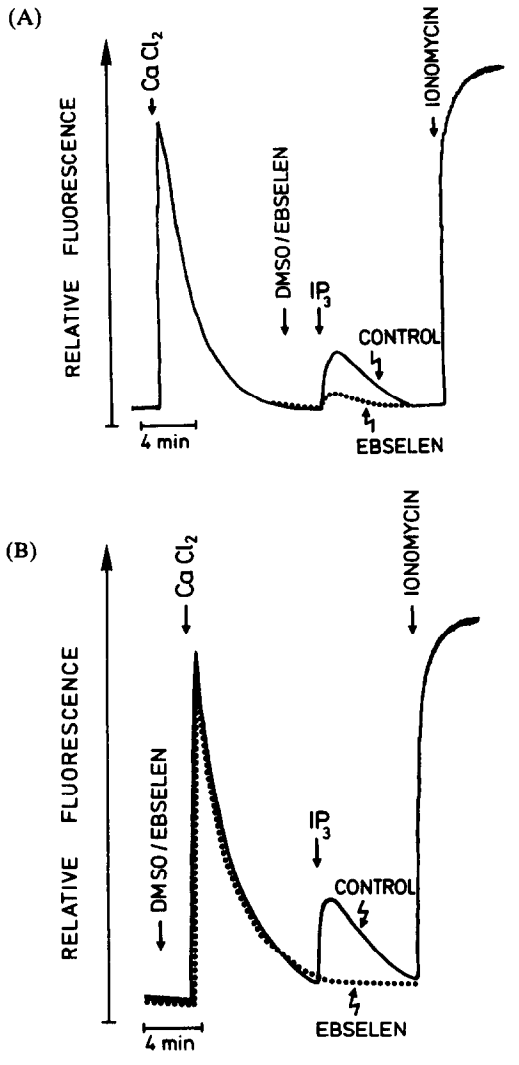

Fig. 5. Modulation of microsomal Ca^{2+} release and uptake by ebselen. (A) Effect of ebselen on microsomal calcium release. (B) Effect of ebselen on microsomal calcium uptake. Calcium measurements using the fura-2 (free acid) method and a microsomal preparation was carried out and calculated as stated in Materials and Methods. Microsomes (0.5 mg/mL) sequestered 10 μM added calcium ($CaCl_2$) immediately. DMSO or 2.5 μM ebselen was introduced as indicated before 3 μM IP_3 was given to induce the calcium release. Ionomycin (10 μM) caused complete depletion of sequestered calcium within seconds.

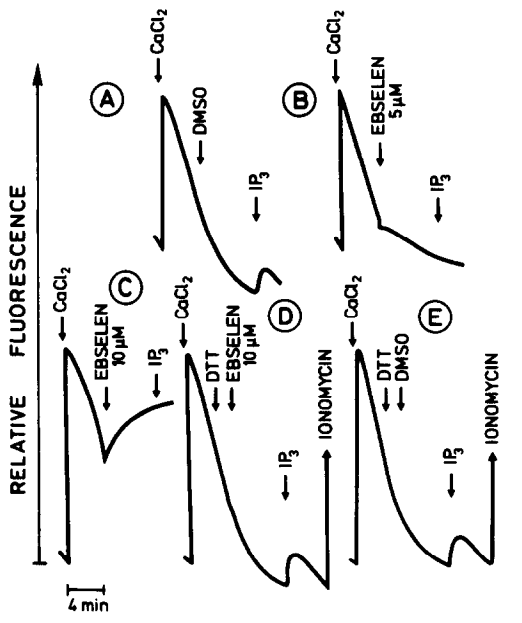

Fig. 6. DTT inhibits calcium release from microsomes caused by high ebselen concentrations. Ebselen (concentrations as given in the figure) at the indicated times influences the microsomal calcium sequestration/release process when assayed with the fura-2 method. Experimental details and concentrations of $CaCl_2$, IP_3 and ionomycin are identical to Fig. 5. DTT (1 mM) was added 1 min before 10 μM ebselen (trace D).

ebselen is unlikely. Again ebselen inhibits the IP_3 induced Ca^{2+} release from microsomes completely and ionomycin within seconds depletes the sequestered calcium. When ATP was rapidly removed from the incubation medium by the addition of glucose and hexokinase, there was a steady loss of sequestered calcium from the microsomes (data not shown). Using this experimental system we were able to observe a more pronounced and sustained IP_3 response because calcium uptake is prevented. Also in this system, similar to that described above, ebselen inhibits the calcium release, showing a dependency upon the concentration used. Ebselen (2.5 μM) completely inhibited the IP_3 response, and 1 μM ebselen resulted in an about 60% inhibition after IP_3 addition (not shown).

When the ebselen concentrations were increased additional effects became obvious. According to the results shown in Fig. 6 it appears that already above 5 μM ebselen the calcium sequestration mechanism becomes affected or that ebselen now induces a passive calcium efflux from microsomes (Fig. 6, trace B compared to trace A). It is also apparent that there is no IP_3 response at this concentration of ebselen.

Increasing the ebselen concentrations to 10 μM (Fig. 6, trace C) resulted in a calcium release from microsomes, although ebselen was added during the calcium sequestration phase. As expected, IP_3 showed no efficacy concerning calcium release when added afterwards. If the same concentration of ebselen was added at the end of the sequestration phase we observed the same phenomenon of ebselen-induced calcium release (data not shown). In the presence of 1 mM DTT (Fig. 6, trace D) ebselen was no longer able to release calcium from the microsomes and we also noticed a normal IP_3 response. Trace E in Fig. 6 represents a control experiment illustrating that the solvent did not affect calcium loading or calcium release from our microsomal preparation. The conclusions from Fig. 6 is that higher concentrations of ebselen are able to release calcium from loaded microsomes, a process

being completely reversible by an excess of thiols like DTT.

DISCUSSION

Blood platelets are not complete cells, however, they possess all major cellular signal transduction pathways and therefore represent excellent models to study such cellular communication systems. The present study is concerned with the action of ebselen which was proposed as an anti-inflammatory drug based on new principles. Although it inhibits cyclooxygenase and lipoxygenase pathways like other non-steroidal anti-inflammatory drugs it is supposed to exert this action by reducing the peroxide tonus required for both groups of enzymes.

That this may not be the only target of ebselen is evident from its inhibition of platelet aggregation in aspirin-treated platelets in which a functional cyclooxygenase can be excluded. The corresponding IC_{50} value is about 9 μM and it was the purpose of our investigation to localize the point of attack of ebselen for this inhibition. The previously suggested blockade of protein kinase C could be confirmed by our experiments with intact cells however it occurs with an IC_{50} value of around 60 μM and hence cannot be a relevant step under our conditions. Probably other protein kinases are affected as well, as can be suggested from the inhibition of myosin-light chain kinase. In contrast the increase of cytosolic Ca^{2+} as an essential event in platelet aggregation is affected by ebselen with an IC_{50} value of about 14 μM. It should be stated in this context that in the case of human platelets a relatively small reduction of the $[Ca^{2+}]_i$ may already result in a complete inhibition of the aggregatory response (Brüne, unpublished). This observation can explain the differences in the IC_{50} values for aggregation and the cytosolic calcium increase, respectively. Since a cytosolic calcium increase represents the final step in the phosphatidylinositol response we measured further parameters. We excluded an interaction with a specific receptor because the aggregation induced by either thrombin, the prostaglandin endoperoxide analogue U46619 or the calcium ionophore A23187 was inhibited. Measurements of phosphatidic acid and the determination of the amount of IP_3 also ruled out an inhibitory effect of ebselen on phospholipase C. The cytosolic calcium concentration is balanced by Ca^{2+} influx and efflux from different compartments. In human platelets the dense tubular system (DTS) represents the main IP_3 sensitive intracellular calcium storage organelle [19]. There is also a calcium influx from the extracellular environment [20] as well as a plasma membrane calcium pump [21] which takes part in the removal of calcium from the cytosol. Our results indicate by using the Mn^{2+} entry and the concomitant fura-2 quenching that ebselen does not affect receptor-operated Ca^{2+} channels.

A more likely explanation of the ebselen effect emerged from studies on calcium uptake and release from a microsomal preparation. To release calcium, IP_3 must bind to receptors that are somehow linked to calcium channels connected with the IP_3-sensitive calcium pool (see Ref. 22 for review). Recent reports demonstrated that the IP_3-induced calcium release is critically dependent on the concentration of external ATP and calcium itself [23–25]. Therefore, our observed IP_3 effect releasing about 25% of the stored calcium and the rapid re-uptake is in accordance with published data. Ebselen at low concentrations inhibited the IP_3-induced calcium release without affecting the calcium uptake system. Compared to the inhibition of the calcium increase using intact cells the concentrations of ebselen are relatively low. This may be explained either by the lipophilicity of ebselen or by the possibility that the compound is sequestered through specific or nonspecific binding sites in intact cells. It should also be noticed in this context that ebselen binds to serum albumin and it is not known whether the free plasma concentration of ebselen is high enough to observe the same inhibition under *in vivo* conditions.

It was interesting to observe a second effect of ebselen when the concentration in our microsomal calcium uptake-efflux system was increased. Above 10 μM ebselen calcium was released from the dense tubular system. The mechanism of this ebselen-induced calcium release may involve a reaction with essential thiol groups at a calcium release channel or at the calcium ATPase. Although a calcium increase must be considered as a proinflammatory and proaggregatory step it seems that platelets are not activated under these conditions (unpublished). It is also very unlikely that the *in vivo* concentrations of ebselen are high enough to account for a calcium release. This also applies for the calcium increase observed in cells exposed to very high concentrations of ebselen. The chemistry of the selenium atom in the molecule would allow it to react directly with free SH-groups under physiological conditions. There is good evidence in literature that sulfhydryl oxidation by reactive disulfides, radicals or heavy metals trigger calcium release from sarcoplasmic reticulum [26–28]. We recently reported that another sulfhydryl group inhibitor ethylmercurithiosalicylate (thimerosal) causes calcium release and subsequent platelet activation, while several other SH reagents inhibited platelet aggregation to a variable extent [29]. In view of mounting evidence for the existence of a critical SH group at or near the release channel, we propose that ebselen triggers calcium release by oxidizing critical SH groups at the dense tubular system. This hypothesis is supported by the observation that the sulfhydryl group reducing agent DTT completely protects against ebselen-induced calcium release.

One can even speculate that the action of ebselen on the IP_3-dependent calcium-release is thiol-sensitive as well since a role of thiol groups and disulphide bridges at the IP_3-recognition sites have been suggested [30, 31].

Very recently Leurs *et al.* [32] reported that ebselen inhibits contractile responses of guinea-pig parenchymal lung strips. The authors speculate that it would be attractive to attribute the effects of ebselen to an interaction with critical thiol groups. Our results now show a direct interaction of ebselen with distinct proteins and point to a direct interaction with a thiol group. The modification of IP_3 induced calcium release and uptake activity would be a new pharmacodynamic mechanism of ebselen action

being different from the postulated role in scavenging hydroperoxides. Under therapeutic conditions not only the peroxide quenching role may be relevant but also the described effects on calcium homeostasis may contribute to the anti-inflammatory properties of ebselen.

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