Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 396-401

INHIBITOR OF ANION TRANSPORT, DIDS, RELEASES Ca^{2+} FROM HEPATIC MICROSOMES

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Received January 5, 1988

SUMMARY: Addition of 4,4'-diisothiocyanostilbene - 2, 2'-disulfonic acid (DIDS) to Ca^{2+} loaded hepatic microsomal vesicles evoked a dose-dependent release of the accumulated Ca^{2+}. Ca^{2+} uptake was also inhibited. The effects of DIDS do not seem to be due to the inhibitions of either Cl^- or proton fluxes. The results indicate that DIDS inhibits Ca^{2+} uptake and releases Ca^{2+} by inhibiting the Ca^{2+}-ATPase and the formation of the phosphorylated intermediate of the enzyme, and that it might interact with a specific site on the vesicle which is involved in the translocation of Ca^{2+} across the microsomal and mitochondrial membranes.

The hepatic microsomal fraction possesses an ATP-dependent Ca^{2+} sequestering system and Ca^{2+}-ATPase activity (1-4). The mechanism by which Ca^{2+} is taken up into the vesicles seems to involve the activation of the Ca^{2+}-ATPase, by the formation of a phosphorylated intermediate (4-6). It has been also reported that in addition to the activation of the Ca^{2+}-ATPase, other factors seem to have an influence on the Ca^{2+} translocation process (7).

The release of Ca^{2+} from microsomes seems to play a key role within the cells in mediating extracellular stimuli, and it has been reported that both GTP and IP_3 cause a release of Ca^{2+} from a heavy microsomal fraction (8). However, the mechanism by which Ca^{2+} is released is not completely understood.

In the course of studies aimed at finding possible factors involved in the regulation of the Ca^{2+} transport process, we examined the effect of the anion transport inhibitor DIDS (9). This agent was shown previously to inhibit the transport of glucose-6-phosphate in the microsomal fraction (10,11). We report here the effect of DIDS on Ca^{2+} release.

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0006-291X/88 $1.50

MATERIALS AND METHODS

Chemicals

ATP, EGTA, Hepes, DTT, DIDS were obtained from Sigma (St Louis, MO). All other reagents were of the highest purity obtainable.

Microsomal preparation

Livers from male fed Sprague-Dawley rats were homogenized in a medium composed of 250 mM sucrose, 20 mM Hepes (pH 7.2), 1 mM EGTA and 1 mM DTT and the microsomal fraction prepared as described previously (12).

Ca^{2+} transport assay

Ca^{2+} transport was measured in a reaction mixture composed of 100 mM KCl, 20 mM Hepes (pH 6.8) and 5 mM $MgCl_2$ with a Ca^{2+} electrode (Orion, Model 93-20, Cambridge, MA) at 37°C. The Ca^{2+} electrode was calibrated using Ca-EGTA buffers of known ionized Ca^{2+} concentrations, which were prepared and standardized using a Ca^{2+} standard solution purchased from Orion Associates Inc. Microsomal protein was approximately 1 mg/ml. Ca^{2+} concentration was adjusted to the desired Ca^{2+} concentration with $CaCl_2$, and uptake was initiated by the addition of ATP to a final concentration of 1 mM.

In some experiments, where the influence of Cl^- on the Ca^{2+} uptake and release was examined, KCl was replaced with either 100 mM K^+-gluconate or 200 mM sucrose. In these experiments, $MgCl_2$ was replaced with $MgSO_4$ and $CaCl_2$ with $CaCO_3$.

ATPase activity

ATPase activity was measured in an incubation mixture composed of 100 mM KCl, 5 mM $MgCl_2$, 20 mM Hepes (pH 6.8), 1 mM NaN_3, 1 mM ouabain, 2 mM EGTA, 1 μM A23187 and about 1 mg protein/ml. For the determination of Ca-stimulated ATPase, $CaCl_2$ was added to give a free Ca^{2+} concentration of 43 μM. The reaction was initiated by adding ATP to give a final concentration of 1 mM and carried out at 37°C for 15 minutes. The reaction was terminated by the addition of cold 8% (w/v) trichloroacetic acid. The protein-free supernatant was assayed for Pi using a colorimetric method (13). Appropriate blanks were used to quantitate nonspecific Pi contamination. Student's t test was employed for statistical analyses.

Phosphorylation experiments

Phosphorylation of microsomal protein by [γ-^{32}P]ATP was carried out in the same incubation medium used for ATPase activity measurement. The phosphorylation reaction, SDS/polyacrylamide-gel electrophoresis and the autoradiographs were carried out as previously described in detail (6).

RESULTS AND DISCUSSION

Fig. 1. demonstrates in a representative experiment the effect of DIDS on Ca^{2+} release from Ca^{2+}-loaded vesicles in KCl-$MgCl_2$-Hepes medium. The response to DIDS was dose-dependent. At the concentration of 500 μM DIDS released, within 3 min, all the Ca^{2+} that had been taken up by the microsomal vesicles. Subsequent addition of the Ca^{2+} ionophore A23187 did not cause any further Ca^{2+} release.

It has been shown previously that ÌM concentrations of DIDS inhibited the activity of the purified and reconstituted Ca^{2+} ATPase from erythrocytes (14) and also the Ca^{2+} ATPase of the sarcoplasmic reticulum (15). The effect of DIDS on basal and Ca^{2+}-stimulated ATPase in hepatic microsomes is shown in Table 1. It is evident that at 50 ÌM DIDS had no inhibitory effect on the basal but a pronounced inhibitory effect on the

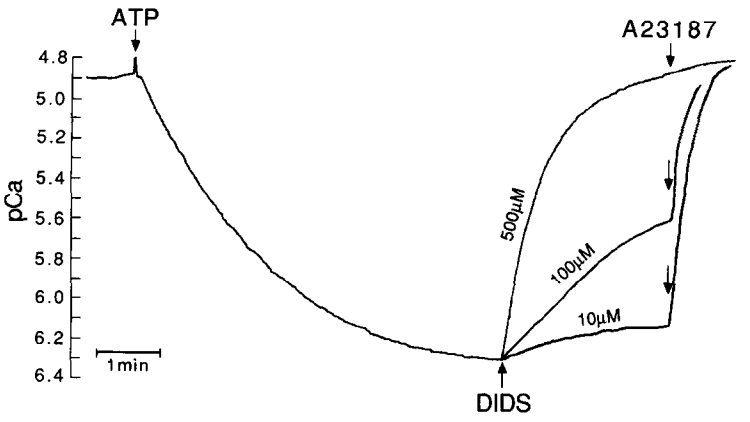

Fig. 1. Effects of DIDS on Ca^{2+} release from microsomes. Ca^{2+} uptake was measured as described in Methods. Ca^{2+} uptake was initiated by adding ATP (1 mM). Other additions were: DIDS (10, 100 and 500 μM as indicated), A23187 (1 μM).

Ca^{2+}-ATPase activity. The inhibition (about 90%) seen with Ca^{2+}-ATPase was statistically significant. Lower concentration of DIDS (10 μM) seemed also to inhibit the Ca^{2+} ATPase but the inhibition was less effective. The effect of DIDS on Ca^{2+}-ATPase was almost maximal at 100 μM.

In accordance with the present observation that DIDS inhibits the Ca^{2+}-ATPase, the apparent ATP-stimulated Ca^{2+} uptake is also inhibited by DIDS (results not shown).

The effect of various concentrations of DIDS on phosphorylation of microsomal protein by [γ-^{32}P]ATP is shown in Fig. 2. In the presence of Ca^{2+} (B in Fig. 2), an approximately 100,000 Mr protein was phosphorylated. This phosphoprotein has been identified to be the phosphorylated

Table 1. Effect of DIDS on basal and Ca^{++}-stimulated ATPase activities

Concentration of DIDS	No. of expts.	ATPase activity* basal	ATPase activity* Ca^{2+}-stimulated	Ca^{2+}-ATPase activity*
0	8	2962 ± 276	3516 ± 301	554 ± 127
10 μM	3	2785 ± 133	3297 ± 146	512 ± 103
50 μM	3	3000 ± 400	3052 ± 395	52 ± 9**
100 μM	3	2709 ± 179	2735 ± 154	26 ± 34**
500 μM	3	2086 ± 223	2058 ± 209	-28 ± 23**

* nmoles Pi/mg protein/hr (mean ± S.D.)
** $p < 0.01$ vs control (no DIDS)

ATPase activities were determined as described in the text.

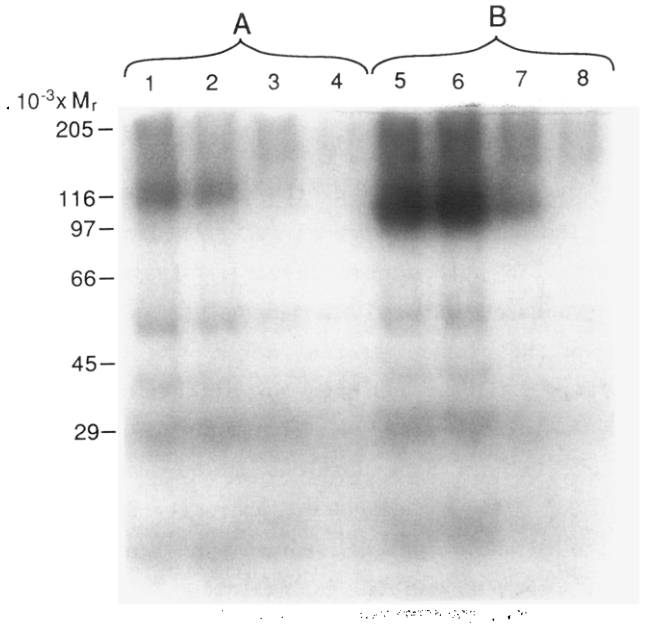

Fig. 2. Effect of DIDS on phosphorylation of microsomal proteins by [γ-^{32}P]ATP. Phosphorylation was carried out for 5 sec at 4°C in the absence (A) and presence (B) of 60 μM $CaCl_2$. Two mM EGTA was included in A. Lanes 1 and 5; no DIDS. DIDS was added to give final concentrations of 10 μM in lanes 2 and 6, 100 μM in lanes 3 and 7, and 500 μM in lanes 4 and 8.

intermediate of Ca^{2+}-ATPase in our previous study (6). As shown in Fig. 2, phosphorylation of the enzyme was inhibited by DIDS at concentrations higher than 10 μM. The concentration range needed for the inhibition of phosphorylation agreed well with that for the inhibition of Ca^{2+}-ATPase activity. Phosphorylation of other proteins were also inhibited in parallel by DIDS. Therefore, a primary effect of DIDS might be the inhibition of phosphorylation of proteins by ATP.

Because DIDS is a known inhibitor of the red cell Cl^- transporter (9), it seems important to clarify whether, in addition to its inhibition of the Ca^{++}-ATPase, DIDS also influences Ca^{2+} translocation indirectly through Cl^- coupled Ca^{2+} movements. Actually, it was reported very recently that Ca^{2+} transport is coupled to anion transport in endoplasmic reticulum prepared from rat pancreatic acinar cells (16).

In order to test this possibility we examined the effect of DIDS on Ca^{2+} release in experimental conditions devoid of Cl^- where KCl was replaced with either K^+-gluconate or sucrose, $MgCl_2$ with $MgSO_4$ and $CaCl_2$ with $CaCO_3$. Ca^{2+} uptake was not reduced in the K^+-gluconate medium, nor was DIDS less effective in releasing Ca^{2+}. In sucrose medium, in accordance with the earlier observation by Moore et al. (1), Ca^{2+} uptake was significantly reduced. However, DIDS was able to release all the Ca^{2+} accumulated (results not shown). These data do not support the notion

that the effect of DIDS on Ca^{2+} release is somehow Cl^- dependent. Therefore, hepatic microsomes may be different from pancreatic microsomes in this aspect.

Another indication that Cl^- transport might not be involved in the effect of DIDS came from studies on the effect of nigericin on microsomal Ca^{2+} release. The hepatic microsomal fraction has been shown to possess an inwardly directed proton pump (17,18). In many cases proton pump activity is associated with Cl^- movements (19). While nigericin was able to collapse the proton gradient across the microsomal membrane as reported previously (17), addition of nigericin did not cause Ca^{2+} release from the microsomal fraction (results not shown). However, according to preliminary data obtained in our laboratory, DIDS causes the release of Ca^{2+} from mitochondria, where the direction of the proton pump is opposite to the microsomal proton pump, and where collapsing the proton gradient with nigericin is followed by massive Ca^{2+} release (results not shown).

In summary, data are presented which show that DIDS causes a rapid, massive Ca^{2+} release from the microsomal fraction. The data do not support the notion that this effect is related to either Cl^- or H^+ fluxes. Rather it shows that DIDS inhibits Ca^{2+} uptake and stimulates Ca^{2+} release by inhibiting the activity of the Ca^{2+}-ATPase, presumably through the inhibition of the formation of phosphorylated intermediate of the enzyme. In addition, as the experiments with mitochondria indicate, DIDS might interact with a site directly involved in Ca^{2+} translocation. The latter possibility may explain the discrepancy between the dose-response relationship for Ca^{2+} release and that for the inhibition of Ca^{2+}-ATPase activity.

ACKNOWLEDGEMENT

Supported by Grant PHS NIH DK 36916.

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