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R 24571: A NEW POWERFUL INHIBITOR OF RED BLOOD CELL Ca^{++}-TRANSPORT ATPase AND OF CALMODULIN-REGULATED FUNCTIONS

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SUMMARY

Compound R 24571 (1-[bis(p-chlorophenyl)methyl]-3-[2,4-dichloro-ß-(2,4-dichlorobenzyloxy)phenethyl]imidazoliniumchloride) is found to be a powerful inhibitor of red blood cell Ca^{++}-ATPase as well as Ca^{++} transport into inside-out red blood cell vesicles with an IC_{50}-value of 0.5 and 2 µM, respectively. The inhibitory action of R 24571 is more specific on the calmodulin-dependent fraction of Ca^{++}-transport ATPase as compared to the basal Ca^{++}-transport ATPase (determined in the absence of calmodulin) and can be antagonized by increasing concentrations of calmodulin in an apparently competitive manner. With respect to other ATPases the action of R 24571 is relatively specific for red blood cell Ca^{++}-transport ATPase. Mg^{++}-ATPase requires a 40 times higher concentration for half-maximal inhibition (IC_{50} = 20 µM) whereas (Na^{+}+ K^{+})-transport ATPase is only slightly affected in the investigated concentration range ($\leq$20 µM).

INTRODUCTION

Low intracellular Ca^{++} concentration in red blood cells (RBCs) is maintained by an active Ca^{++}-transport mechanism whose biochemical expression presumably is the Ca^{++}-ATPase (1). The Ca^{++}-transport ATPase of RBCs is regulated by the ubiquitous Ca^{++}-binding protein, calmodulin, in dependence on the cytoplasmic Ca^{++} concentration (2,3). Besides RBC Ca^{++}-transport ATPase calmodulin mediates control of a number of Ca^{++}-dependent cellular functions (3).

The search for a specific and potent inhibitor of RBC Ca^{++}-transport ATPase has thus far been unsuccessful (4). The available inhibitors for RBC Ca^{++}-transport ATPase are far from being satisfactory. Vanadate, the lanthanides, quercetin and suramin-Na are rather unspecific in that

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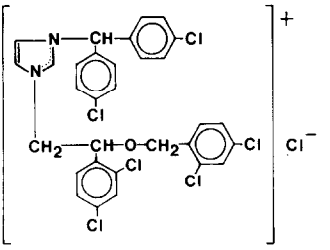

Fig. 1. Structure of R 24571.

they also inhibit other membrane ATPases such as $(Na^+ + K^+)$-transport ATPase (4). Ruthenium red gives undesirably complex effects (5) whereas the phenothiazines, butyrophenones (6,7) and vinblastine (8) preferentially inhibit the calmodulin-dependent fraction of RBC Ca^{++}-transport ATPase by interaction with calmodulin.

Thus, in searching for a highly specific and potent inhibitor of Ca^{++}-transport ATPase, we examined the effects of R 24571 on Ca^{++}-transport ATPase, $(Na^+ + K^+)$-transport ATPase and Mg^{++}-ATPase in human RBC membranes. The structure of R 24571, a highly lipophilic substance, is shown in Fig. 1. A preliminary report of our investigations has been presented (9).

MATERIALS AND METHODS

All reagents were of highest purity available. R 24571 kindly was provided by Janssen Pharmaceutica, Beerse, Belgium. The lipophilic compound R 24571 was dissolved in dimethyl sulfoxide (DMSO) and added to the assay medium under vigorous mixing. The final concentration of DMSO in the assay medium including the controls was in all cases 0.5% (v/v). The inhibition studies were performed in plastic tubes since R 24571, due to its lipophilicity, easily sticks to glass surfaces in aqueous solutions. As a consequence of its hydrophobic character the highest drug concentration of R 24571 that can be achieved in aqueous medium at pH 7.0 is about 20 μM.

Preparation of calmodulin and of calmodulin-deficient RBC membranes: Bovine brain calmodulin was prepared as described by Watterson et al. (10). Membrane-bound human RBC membranes were prepared according to the iso-osmotic freeze-haemolysis procedure described by Gietzen et al. (6).

Preparation of calmodulin-deficient RBC inside-out vesicles: Human RBCs were washed in isotonic NaCl solution and lysed at 2°C in a fifteenfold volume of a medium consisting of 15.25 mM Tris-Cl, 1 mM Tris-EGTA, pH 7.75. The mixture was centrifuged at 28 000 g under refrigeration for 20

min and the membranes were washed four times in the same solution. Before the third and the fourth centrifugation they were incubated for 30 min at 37°C. Then they were once washed in: 15.25 mM Tris-Cl (pH 7.75), 5 μM $CaCl_2$. To initiate vesiculation the white membranes were diluted in a twentyfold volume of a solution containing 0.5 Tris-Cl (pH 8.5) and 50 μM dithiothreitol. The mixture first was incubated for 30 min at 0°C and then for additional 15 min at 37°C before the vesicles were pelleted at 28 000 g in the cold for 20 min. The concentrated vesicle suspension was homogenized by passing it four times through a 26 gauge hypodermic needle, pelleted again and resuspended in 140 mM KCl, 20 mM Tris-Cl (pH 7.4) at 0°C to give a final protein concentration of about 2.5 mg/ml.

Assay of ATPase activities: ATPase activities were determined at 37°C as described previously (11). In the case of Ca^{++}-transport ATPase the reaction was monitored continuously for 8 min whereas in the case of Mg^{++}-ATPase and $(Na^+ + K^+)$-transport ATPase the reaction was followed discontinuously over a period of 90 min. The assay medium contained, in a final incubation medium of 10 ml, 60 μg membrane protein/ml. The medium for Ca^{++}-transport ATPase consisted of 10 mM Tris-maleate buffer (pH 7.0), 100 mM KCl, 0.2 mM ouabain, 1 mM ATP, 2 mM $MgCl_2$ and 36 μM Ca^{++} (as a Ca^{++}-Mg^{++}-EDTA buffer (12)). Ca^{++} free controls contained instead of the Ca^{++}-Mg^{++}-EDTA buffer 0.4 mM Mg^{++}-EGTA. These controls yielded simultaneously the Mg^{++}-ATPase activity. The medium for $(Na^+ + K^+)$-transport ATPase consisted of 100 mM NaCl, 10 mM KCl, 30 mM imidazole-Cl, 4 mM $MgCl_2$, 0.5 mM Tris-EGTA, 2 mM ATP, with or without 0.2 mM ouabain. Before starting the reaction by addition of ATP the RBC membranes were preincubated with R 24571 for 10 min at 37°C in the case of Mg^{++}-ATPase and $(Na^+ + K^+)$-transport ATPase whereas in the case of Ca^{++}-transport ATPase the RBC membranes were first preincubated for 10 min with the drug followed by additional 10 min in the absence or presence of calmodulin (0.4 μg/ml).

Ca^{++}-uptake into inside-out RBC vesicles: Ca^{++}-transport measurements were carried out as described before (5). Before starting the transport process with ATP the vesicles were preincubated for 20 min with R 24571 and for 10 min with Ca^{++} at 37°C in a medium consisting of 130 mM KCl, 20 mM imidazole-Cl, 2 mM $MgCl_2$, 0.2 mM EGTA, 0.19 mM $CaCl_2$ (+ 0.1 μCi $^{45}Ca^{++}$/ml) yielding a free Ca^{++} concentration of 4.65 μM, 2 mM Mg-ATP, pH 7.0. The assay medium contained 39 μg vesicle protein/ml and when present 0.1 μg calmodulin/ml. Samples (1 ml) were taken at 0,3 and 6 min and filtered immediately through Millipore filters with a pore diameter of 0.45 μm which retained all protein.

RESULTS

The effects of R 24571 on human RBC $(Na^+ + K^+)$-transport ATPase as well as on the basal (determined in the absence of calmodulin) and calmodulin-dependent fraction of Ca^{++}-transport ATPase are shown in Fig. 2. R 24571 is a highly potent inhibitor of RBC Ca^{++}-transport ATPase with an IC_{50} of 0.5 μM for the total Ca^{2+}-transport ATPase activity. In contrast Mg^{++}-ATPase (IC_{50}= 20 μM; see Table 1) and $(Na^+ + K^+)$-transport ATPase (16% inhibition at 20 μM) are far less susceptible to the drug. The inhibitory action of R 24571 on Ca^{++}-transport ATPase is more specific on the calmo-

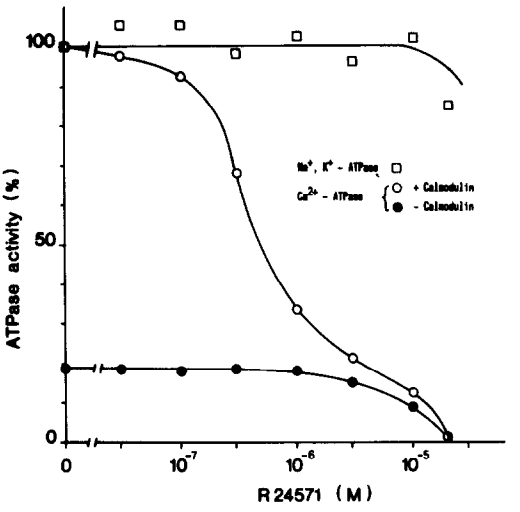

Fig. 2. Effects of R 24571 on $(Na^+ + K^+)$-transport ATPase and Ca^{++}-transport ATPase activities. Ca^{++}-transport ATPase activity is related to the calmodulin (0.4 µg/ml)-stimulated enzyme in the presence of 0.5% DMSO and in the absence of the drug (100% activity = 50-70 nmoles/mg protein·min). $(Na^+ + K^+)$-transport ATPase activity is related to the enzyme activity in the presence of 0.5% DMSO and in the absence of R 24571 (100% activity = 7-10 nmoles/mg protein·min). Each point represents the mean of four determinations.

dulin-dependent fraction (IC_{50}= 0.35 µM) as compared to the basal fraction (IC_{50}= 10 µM). This fact also can be seen from the biphasic decay of the Ca^{++}-transport ATPase activity in the presence of calmodulin with increasing concentrations of R 24571.

Table 1: Inhibitory effect of R 24571 on red blood cell ATPase activities

ATPase	Concentration for half-maximal inhibtion (µM)
Ca^{++}-transport ATPase	0.5
basal (in the absence of calmodulin)	10
calmodulin-dependent fraction	0.35
Mg^{++}-ATPase	20
$(Na^+ + K^+)$-transport ATPase	>20 (16)*

IC_{50}-values were obtained graphically from inhibition curves. The inhibition studies were performed for each ATPase with 60 µg membrane protein/ml.

*The value in parentheses indicates the percentage of inhibition at the highest drug concentration used.

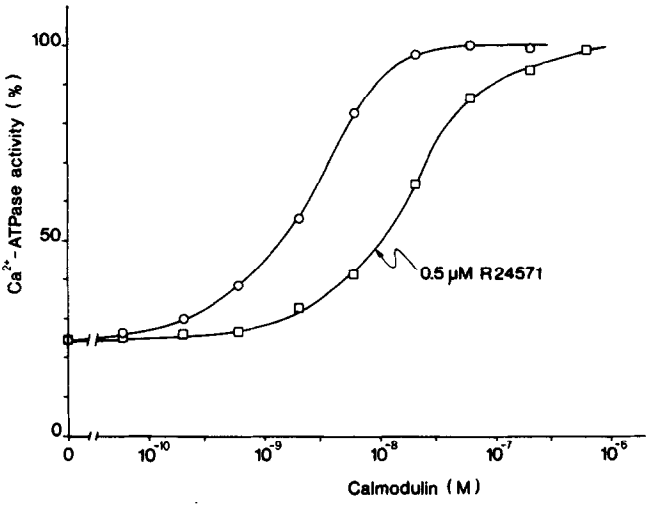

Fig. 3. Antagonism of calmodulin-stimulated Ca^{++}-transport ATPase activity by R 24571. Basal Ca^{++}-transport ATPase is stimulated by increasing amounts of calmodulin in the absence (○) and presence (□) of the drug. Sample with 60 nM calmodulin, 0.5% DMSO and no R 24571 = 100% activity (= 50-70 nmoles/mg protein·min). Each point is the mean of four determinations.

Fig. 3 gives evidence that R 24571 with a high specificity antagonizes the activation of Ca^{++}-transport ATPase by calmodulin in an apparent competitive manner since increasing concentrations of calmodulin completely reverse the inhibition caused by the drug.

Fig. 4 demonstrates the inhibition of Ca^{++} transport into inside-out RBC vesicles in the presence and absence of added calmodulin as a function of the R 24571 concentration. Total Ca^{++} transport (basal and calmodulin-dependent fraction) is half-maximally inhibited at 2 µM. The higher specificity of R 24571 for the calmodulin-dependent fraction is less obvious for transport (Fig. 4) as for ATPase activity (Fig. 2). This might be due to the fact that inside-out vesicles are much less depleted in calmodulin as compared to disrupted RBC membranes.

It is demonstrated in Fig. 5 that the IC_{50}-values are dependent on the amount of RBC membranes used in the assay medium. This is not surprising since R 24571 is highly lipophilic and therefore soluble in the hydrocar-

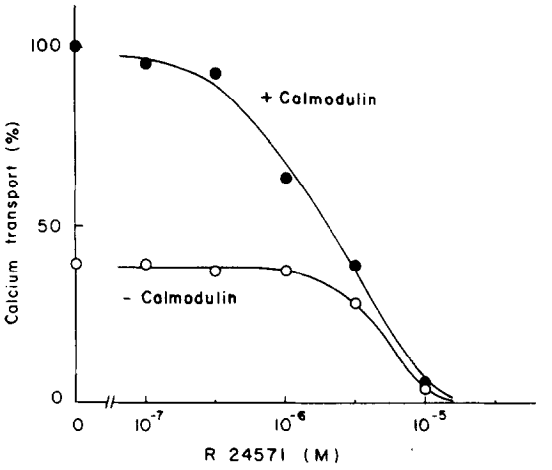

Fig. 4. Inhibition of Ca^{++} transport into inside-out RBC vesicles by R 24571 in the presence (0.1 µg/ml) or absence of calmodulin. Ordinate: relative initial rate (0-6 min after adding ATP) of $^{45}Ca^{++}$ uptake into vesicles (100% activity = 5.4 nmoles Ca^{++}/mg protein·min).

bon part of the membrane leading to a decrease of the free concentration of the drug. Out of this reason in each case equal amounts of RBC membranes were used in order to determine differences in the affinities of the ATPases for R 24571.

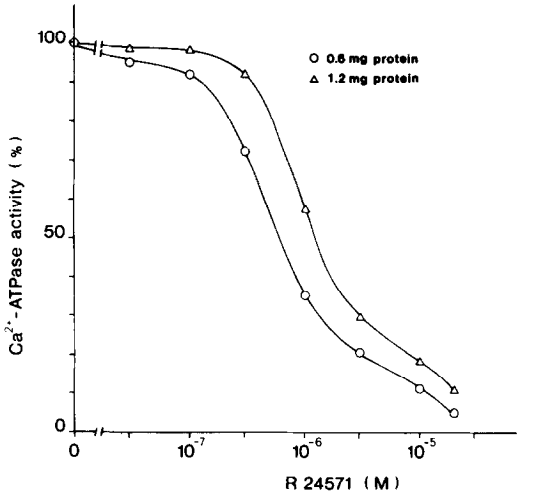

Fig. 5. Inhibition of Ca^{++}-transport ATPase by R 24571 determined for two different membrane protein concentrations. Sample with 0.4 µg calmodulin/ml, 0.5% DMSO and no R 24571 = 100% activity (=50-70 nmoles/mg protein·min). Each point represents the mean of at least two determinations.

The concentrations of R 24571 producing 50% inhibition (IC_{50}) of the Ca^{++}-transport ATPase, Mg^{++}-ATPase and (Na^{+}+ K^{+})-transport ATPase are summarized in Table 1.

DISCUSSION

The present results demonstrate that R 24571 is a highly potent inhibitor of RBC Ca^{++}-ATPase and Ca^{++} transport with IC_{50}-values of 0.5 and 2 μM, respectively. Nearly 100% inhibition of both phenomena can be achieved with 10 to 20 μM of the drug. With respect to other ATPases the action of R 24571 on RBC Ca^{++}-transport ATPase is rather specific since the Ca^{++}-transport ATPase displays a 40 times higher affinity for the drug as compared to Mg^{++}-ATPase and the difference of affinity is even higher when Ca^{++}-transport ATPase is compared with (Na^{+}+ K^{+})-transport ATPase which is only slightly inhibited at the highest possible drug concentration. We suggest that the relatively high potency and specificity of R 24571 as an inhibitor of RBC Ca^{++}-transport ATPase may allow its use as a tool to discriminate between the ATPase activities of RBC membranes.

The inhibitory action of R 24571 on Ca^{++}-transport ATPase is more specific on the calmodulin-dependent fraction as compared to the basal fraction of the ATPase activity. In addition it is shown that R 24571 antagonizes the activation of RBC Ca^{++}-transport ATPase by calmodulin in an apparently competitive fashion. We found that R 24571 under the same conditions exhibits a 26 times higher potency (cf. 6) in inhibiting the calmodulin-dependent fraction of RBC Ca^{++}-transport ATPase as compared to the commonly used inhibitor of calmodulin-regulated functions, trifluoperazine. Van Belle (13) could show that R 24571 also inhibits the activation of brain phosphodiesterase by calmodulin with a 500 times higher potency than trifluoperazine. Thus R 24571 seems to be the most powerful inhibitor of calmodulin-regulated processes that has been described hitherto. Since R 24571 - in contrast to other calmodulin inter-

acting agents (phenothiazines or butyrophenones) - has no affinity for dopamine receptors at concentrations that completely inhibit calmodulin-regulated functions (Van Belle, personal communication) R 24571 is proposed to be a useful tool for studying the possible involvement of calmodulin in dopaminergic transmission (14).

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REFERENCES

1. Schatzmann, H.J. (1973) J. Physiol. (London) 235, 551-569
2. Vincenzi, F.F. (1978) Ann. N.Y. Acad. Sci. 307, 229-231
3. Cheung, W.Y. (1980) Science 207, 19-27
4. Schatzmann, H.J. (1981) in Membrane Calcium Transport (Carafoli, E., ed.) Academic Press, London, in press
5. Wüthrich, A. and Schatzmann, H.J. (1980) Cell Calcium 1, 21-35
6. Gietzen, K., Mansard, A. and Bader, H.(1980) Biochem. Biophys. Res. Commun. 94, 674-681
7. Levin, R.M. and Weiss, B. (1980) Neuropharmacol. 19, 169-174
8. Gietzen, K. and Bader, H. (1980) IRCS Med. Sci. 8, 396-397
9. Gietzen, K. and Mansard, A. (1981) Naunyn-Schmied. Arch. Pharmacol. 316 (Suppl.), R 43
10. Watterson, D.M., Harrelson, W.G., Keller, P.M., Sharief, F. and Vanaman, T.C. (1976) J. Biol. Chem. 251, 4501-4513
11. Arnold, A., Wolf, H.U., Ackermann, B.P. and Bader, H. (1976) Anal. Biochem. 71, 209-213
12. Wolf, H.U. (1973) Experientia 29, 241-249
13. Van Belle, H. (1981) Biochem. Soc. Trans. 9, 133 P
14. Hanbauer, I. and Phyall, W. (1980) Adv. Biochem. Psychopharmacol. 24, 133-138