Biochemical Pharmacology, Vol. 26, pp. 1667–1670. Pergamon Press, 1977. Printed in Great Britain.

EFFECTS OF RESERPINE AND SEROTONIN ON ADENINE NUCLEOTIDE METABOLISM AND GLUCOSE OXIDATION OF WASHED RABBIT PLATELETS

HANS-JOACHIM REIMERS, RAELENE L. KINLOUGH-RATHBONE, MARIAN A. PACKHAM and J. FRASER MUSTARD

Department of Pathology, McMaster University, Hamilton, Ontario, and Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada

(*Received* 8 *July* 1976; *accepted* 17 *December* 1976)

Abstract—Reserpine inhibits the transfer of ATP from the metabolically active pool into the releasable pool (amine storage organelles) of washed rabbit platelets. Since reserpine is known to interfere with oxidative phosphorylation of isolated mitochondria, possible effects of reserpine on the energy metabolism of intact platelets were examined with the aim of determining whether or not such effects could explain the reduced transfer of metabolic ATP into the releasable pool of ATP. Reserpine caused a small increase in [^{14}C]hypoxanthine and [^{14}C]inosine accumulation in a suspension of washed rabbit platelets prelabeled with [^{14}C]adenosine or [^{14}C]adenine. Reserpine also caused increased oxidation of [^{14}C]glucose. Serotonin mimicked these effects of reserpine, and imipramine inhibited the serotonin-induced as well as the reserpine-induced increases in [^{14}C]hypoxanthine and [^{14}C]inosine formation and glucose oxidation. However, imipramine did not prevent the reserpine-induced inhibition of [^{14}C]ATP transfer from the metabolically active pool into the releasable pool. It is concluded that the effects of reserpine on energy metabolism are at least partially attributable to the increased demand for energy for the uptake of serotonin, liberated from the platelets during incubation with reserpine, and not to uncoupling of oxidative phosphorylation. Furthermore, the small decrease of the metabolically active ATP pool upon incubation of platelets with reserpine cannot explain the reduced transfer of ATP from the metabolic pool into the amine storage granule pool.

Reserpine interferes with the transport and storage of monoamines in nervous tissue [1] and cells such as blood platelets [2–5]. In a previous paper [6] we provided evidence that reserpine also interferes with the transfer of ATP from the metabolically active pool into the releasable ATP pool which is considered to be located in the amine storage organelles of platelets [4]. Since reserpine has been reported to uncouple oxidative phosphorylation in isolated liver and kidney mitochondria [7], we have now investigated some effects of reserpine on platelet energy metabolism with the aim of determining whether or not these effects are responsible for the reduced transfer of metabolic ATP into the releasable pool of ATP.

Reserpine causes the liberation of serotonin from blood platelets [3, 4]. This serotonin is available for reuptake across the platelet plasma membrane—a process that is not inhibited by reserpine [5]. Serotonin uptake across the platelet plasma membrane is thought to occur by an active, energy-requiring mechanism [3]. We therefore examined whether this energy-requiring process caused increased glucose oxidation and/or increased conversion of platelet adenosine triphosphate to hypoxanthine in the presence of reserpine or added serotonin.

MATERIALS AND METHODS

The source and preparation of materials were the same as those described previously [5, 6]. Additional materials used in these experiments were: oligomycin (15% oligomycin A, 85% oligomycin B) was purchased from Sigma Chemical Co., St. Louis, MO. It was dissolved in 95% ethanol and diluted in glucose-free Tyrode solution. D-[6-^{14}C]glucose was obtained from New England Nuclear Corp., Boston, MA.

Preparation of suspensions of washed platelets. Suspensions of washed platelets were prepared as described by Ardlie *et al.* [8, 9]. Platelets were incubated with [8-^{14}C]adenosine or [U-^{14}C]adenine to label their metabolic adenine nucleotides [10] as described previously [6].

Formation of adenosine triphosphate metabolites. After being labeled with radioactive adenosine or adenine and resuspended in fresh Tyrode solution containing 0.35% albumin and 5 mM HEPES, (*N*-2-hydroxyethyl-piperazine-*N*′-2-ethane sulfonic acid) buffer (pH 7.35), the platelets were kept at 37° for up to 8 hr in conical plastic tubes in the presence or absence of the drug to be tested. At indicated time intervals, 1-ml aliquots were removed from the platelet suspension, and [^{14}C]ATP and its radioactive metabolites were determined in 3% perchloric acid extracts of the platelet suspension. The labeled compounds in the supernatant fluid obtained by centrifugation of the platelet suspension (12,000 *g*, 45 sec) were also determined at the same time after treatment for 3 min with Tyrode solution (control) or thrombin (0.45 units/ml). The detailed methods have been described previously [6, 11].

Adenylate energy charge. From the concentration of [^{14}C]ATP, [^{14}C]ADP and [^{14}C]AMP, the adenylate energy charge of the platelets was calculated as described by Mills [12]. Values were corrected for releasable [^{14}C]ATP, [^{14}C]ADP and [^{14}C]AMP [13].

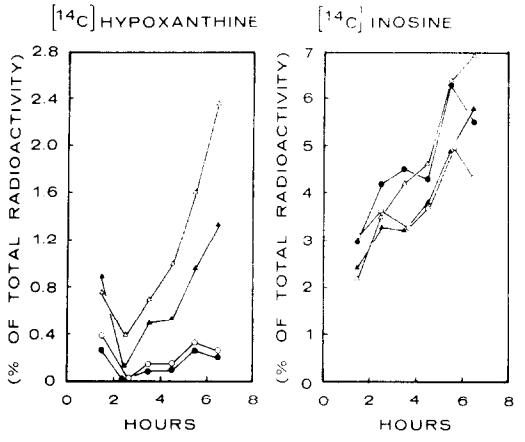

Fig. 1. Effect of reserpine and imipramine on formation of [^{14}C]hypoxanthine (left) and [^{14}C]inosine (right) in a suspension of washed rabbit platelets preincubated with [^{14}C]adenine to label the metabolic adenine nucleotide pool. The platelet suspension was kept at 37° and 1-ml samples of the platelet suspension were removed at the indicated time intervals. Perchloric acid extracts of the total platelet suspension and the supernatant fluid were chromatographed on paper to separate the adenine nucleotides and their metabolites. Practically all of the [^{14}C]hypoxanthine and [^{14}C]inosine was found in the supernatant fluid. This was one of three similar experiments. Key: △——△, reserpine (2 μM); ▲——▲, reserpine (2 μM) + imipramine (20 μM); ○——○, imipramine (20 μM); and ●——●, control.

Glucose oxidation. Glucose oxidation was measured by determining the formation of $^{14}CO_2$ from [6-^{14}C]glucose using a method described previously [14].

RESULTS AND DISCUSSION

When washed platelets suspended in Tyrode solution containing 0.35% albumin and 5 mM HEPES were kept for several hours at 37°, a small amount of inosine and hypoxanthine accumulated in the suspending medium. Figure 1 shows that about 1 per cent of the metabolic ATP (labeled with [^{14}C]adenine or [^{14}C]adenosine) was converted per hour to [^{14}C]inosine. The [^{14}C]hypoxanthine accumulating during 7 hr of incubation was negligible. Reserpine (2 μM), however, caused the accumulation of a significant amount of [^{14}C]hypoxanthine after prolonged incubation. Reserpine had little effect on the accumulation of [^{14}C]inosine.

Figure 2 shows that reserpine (2 μM) also increased the production of $^{14}CO_2$ from [6-^{14}C]glucose. In contrast to the increase of accumulation of [^{14}C]hy-

Table 1. Effect of reserpine on adenylate energy charge of platelets*

Time of incubation (hr)	Adenylate energy charge†	
	Control platelets	Reserpine-treated platelets
2	0.930 ± 0.010	0.936 ± 0.012‡
3	0.946 ± 0.010	0.936 ± 0.015‡
5	0.948 ± 0.010	0.944 ± 0.009‡
7	0.951 ± 0.029	0.941 ± 0.011‡

* Means and S. D. of five experiments.

† The adenylate energy charge was calculated from the distribution of non-releasable [^{14}C]ATP, [^{14}C]ADP and [^{14}C]AMP in suspensions of washed rabbit platelets labeled with [^{14}C]adenosine or [^{14}C]adenine. The amounts of [^{14}C]ATP, [^{14}C]ADP and [^{14}C]AMP that could be released with a high concentration of thrombin (0.45 units/ml) were subtracted from the amounts of [^{14}C]ATP, [^{14}C]ADP and [^{14}C]AMP that were found in the total platelet suspension.

‡ Differences between the means of control platelets and reserpine-treated platelets were not significant at the 95 per cent confidence level at any time.

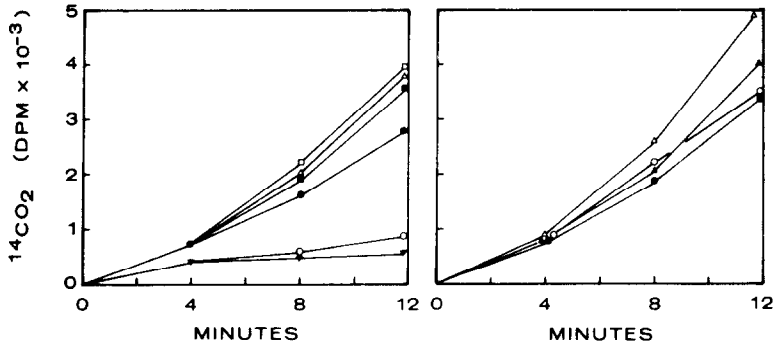

Fig. 2. Production of $^{14}CO_2$ from [6-^{14}C]glucose (167 μM, 0.2 μCi) by washed rabbit platelets suspended in a modified Tyrode–albumin solution containing 5 mM HEPES (pH 7.35) without glucose (platelet count: 1 × 10^6/μl). Left panel: effect of reserpine and oligomycin on $^{14}CO_2$ production. Key: ●——●, Tyrode (control); △——△, reserpine (2 μM); ▼——▼, oligomycin (0.1 μM; added in 10 μl of 95% ethanol to give a final concentration of 0.63% ethanol); ○——○, reserpine (2 μM) + oligomycin (0.1 μM); □——□, reserpine (2 μM) + ethanol (0.63%); and ■——■, ethanol (0.63%, control). Tyrode, ethanol and oligomycin were added to the platelet suspension together with [6-^{14}C]glucose at the beginning of the experiment. Reserpine was added 4 min after the commencement of the incubation of the platelet suspension with [6-^{14}C]glucose. Right panel: effect of reserpine and imipramine on $^{14}CO_2$ production. Key: ●——●, Tyrode (control); △——△, reserpine (2 μM); ○——○, imipramine (20 μM); ▲——▲, reserpine (2 μM) + imipramine (20 μM). All additions were made at the same time as the incubation of the platelet suspension with [6-^{14}C]glucose was commenced. This was one of two experiments with similar results.

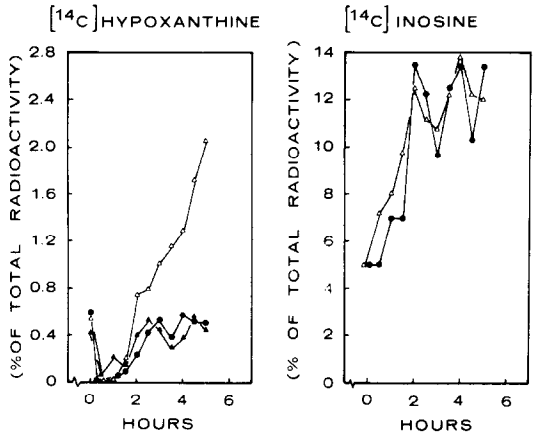

Fig. 3. Effect of serotonin and imipramine on formation of [^{14}C]hypoxanthine (left panel) and [^{14}C]inosine (right panel) in a suspension of washed platelets preincubated with [U-^{14}C]adenine to label the metabolic adenine nucleotide pool. The platelet suspension was kept at 37° and 1-ml samples of the platelet suspension were removed at the indicated time intervals. Perchloric acid extracts of the total platelet suspension and the supernatant fluid were chromatographed on paper to separate the adenine nucleotides from their metabolites. Practically all of the [^{14}C]hypoxanthine and [^{14}C]inosine was found in the supernatant fluid. Key: △——△, serotonin (initial concn 123 μM); ▲——▲, serotonin (initial concn 123 μM) + imipramine (20 μM); and ●——●, control. This was one of two similar experiments.

poxanthine upon prolonged incubation of a platelet suspension with reserpine, the effect of reserpine on $^{14}CO_2$ formation from [6-^{14}C]glucose was apparent immediately. Reserpine did not prevent the oligomycin-induced inhibition of $^{14}CO_2$ production (Fig. 2). An increase in platelet O_2 consumption [15–18] and hypoxanthine [19, 20] and CO_2 [14] formation can be observed during energy-requiring processes such as platelet shape change, aggregation and the release reaction. Increased hypoxanthine formation can also be found after inhibition of platelet glycolysis and oxidative phosphorylation with antimycin and deoxyglucose [21]. Our findings that oligomycin-induced inhibition of $^{14}CO_2$ production was not prevented by reserpine (2 μM) and that the adenylate energy charge of the platelets was not significantly altered by reserpine (Table 1) indicated that ADP rephosphorylation was not measureably impaired and that reserpine at the concentration used did not uncouple oxidative phosphorylation in platelets. This is in agreement with the earlier finding by Century and Horwitt [22] that reserpine had only a slight effect on oxidative phosphorylation in rat brain homogenates at approximately ten times higher concentrations than those used here. Furthermore, the increased production of hypoxanthine only became apparent several hours after the addition of reserpine. This may indicate that the increased production of [^{14}C]hypoxanthine may be due to a process that requires increasing amounts of energy with time. In this regard, it should be pointed out that reserpine causes the liberation into the suspending fluid of endogenous platelet serotonin [4] which is then available for reuptake across the platelet plasma membrane by an active, i.e. energy-requiring, process [3]. It is also possible that initially little or no hypoxanthine accumulated in the platelet suspension (in spite of increased energy demands and

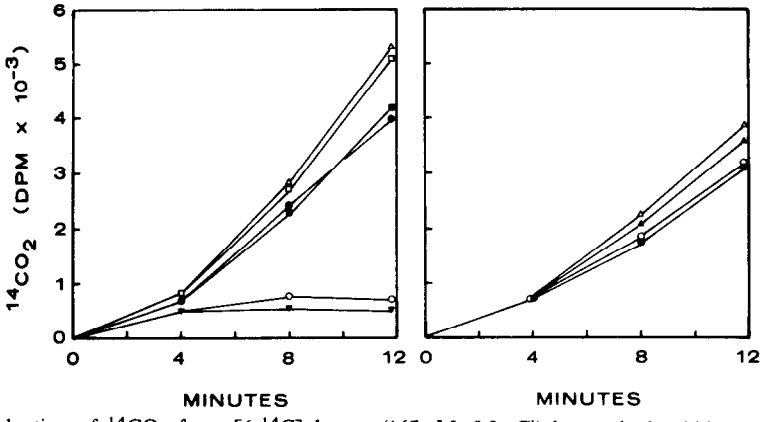

Fig. 4. Production of $^{14}CO_2$ from [6-^{14}C]glucose (167 μM, 0.2 μCi) by washed rabbit platelets suspended in a modified Tyrode–albumin solution containing 5 mM HEPES (pH 7.35) but no unlabeled glucose (platelet count: 1 × 10^6/μl). Left panel: effect of serotonin and oligomycin on $^{14}CO_2$ production. Key: ●——●, Tyrode (control); △——△, serotonin (123 μM); ▼——▼, oligomycin (0.1 μM; added in 10 μl of 95% ethanol to give a final concentration of 0.63% ethanol); ○——○, serotonin (123 μM) + oligomycin (0.1 μM); □——□, serotonin (123 μM) + ethanol (0.63%); and ■——■, ethanol (0.63%, control). Tyrode, ethanol and oligomycin were added to the platelet suspension together with [6-^{14}C]glucose at the beginning of the experiment. Serotonin was added 4 min after the incubation of the platelet suspension with [6-^{14}C]glucose was commenced. Right panel: effect of serotonin and imipramine on $^{14}CO_2$ production. Key: ●——●, Tyrode (control); △——△, serotonin (123 μM); ○——○, imipramine (10 μM; 40 μM imipramine examined at the same time gave similar results); and ▲——▲, serotonin (123 μM) + imipramine (10 μM; 40 μM examined at the same time gave similar results). All additions were made 4 min after the commencement of the incubation with [6-^{14}C]glucose. These results were representative of three experiments with similar results. In these experiments, imipramine (10 or 40 μM) did not cause complete inhibition of the increased $^{14}CO_2$ production in the presence of 123 μM serotonin. In experiments with 12.3 μM serotonin, imipramine (20 μM) completely blocked the increased $^{14}CO_2$ production.

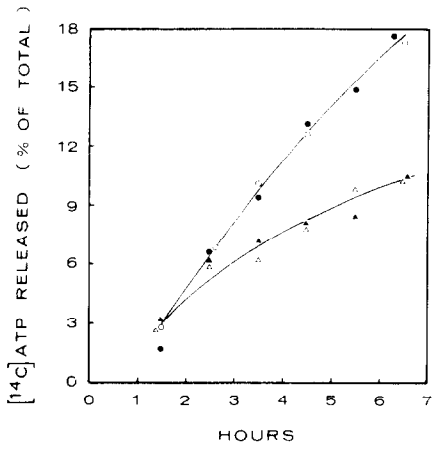

Fig. 5. Effect of reserpine and imipramine on time-dependent increase of [^{14}C]ATP that could be released from washed rabbit platelets prelabeled with [U-^{14}C]adenine. At the times indicated, 1-ml samples of the platelet suspension were removed. Perchloric acid extracts were prepared from the platelet suspensions and from the supernatant fluids after exposure of the platelet suspension to Tyrode solution (control) or thrombin (0.45 units/ml). [^{14}C]ATP was separated from other adenine nucleotides and their metabolites by paper chromatography. The percentage of the total platelet-bound [^{14}C]ATP released by thrombin is plotted. Values were corrected for [^{14}C]ATP found in the supernatant fluid of the sample exposed to Tyrode solution. Key: ●——●, Tyrode; △——△, reserpine (2 μM); ○——○, imipramine (20 μM); and ▲——▲, reserpine (2 μM) + imipramine (20 μM). This was one of two similar experiments.

increased hypoxanthine formation) since hypoxanthine can be reincorporated into adenine and guanine nucleotides by a salvage pathway [23]. This is apparent from Fig. 1, which shows that within hr 1 of observation, hypoxanthine that had been formed during centrifugation and resuspension of the platelets disappeared again from the platelet suspension. However, the reasons for the incomplete reincorporation of hypoxanthine into the adenine and guanine nucleotides upon prolonged incubation of the platelets remain unexplained from the present experiments.

The hypothesis that the reserpine-induced increase in [^{14}C]hypoxanthine accumulation and the increase in $^{14}CO_2$ production from [6-^{14}C]glucose are mediated by serotonin liberated from the platelets was examined in the following experiments. Serotonin at a concentration (123 μM) that was not completely incorporated into the platelets during the course of the experiment caused an increased accumulation of [^{14}C]hypoxanthine in the platelet suspension (Fig. 3) and increased the formation of $^{14}CO_2$ from [6-^{14}C]-glucose (Fig. 4). The accumulation of [^{14}C]hypoxanthine was delayed as it was in the case of reserpine, indicating that it takes some time before the hypoxanthine salvage pathway is overloaded under the conditions of the present experiments. Both effects of serotonin were diminished by the addition of 10–40 μM imipramine, a drug known to inhibit active serotonin uptake across the platelet plasma membrane [3, 4] (Figs. 3 and 4). Imipramine (20 μM) also reduced the reserpine-induced increase in $^{14}CO_2$ production (Fig. 2) and the reserpine-induced increase in [^{14}C]hypoxanthine formation (Fig. 1).

Although imipramine (20 μM) reduced the reserpine-induced $^{14}CO_2$ production and [^{14}C]hypoxanthine formation, it did not alter the reserpine-induced inhibition of [^{14}C]ATP transfer from the metabolic pool into the releasable pool (Fig. 5). This indicates that the previously observed inhibition of [^{14}C]ATP transfer by reserpine [6] is not due to changes in the concentration of [^{14}C]ATP in the metabolic pool. It is more likely that the effect of reserpine on [^{14}C]ATP transfer is exerted at the platelet granule membrane, whereas the other observed metabolic effects of reserpine may be due primarily to reserpine-induced liberation of platelet serotonin.

Acknowledgements—We are grateful for the expert technical assistance of Miss S. Turner, Mr. A. Chahil and Mr. F. Skerlan. This work was supported by Medical Research Council of Canada grants MT 1309 and MT 2629 and by the award of a Fellowship of the Ontario Heart Foundation to H.-J. Reimers. R. L. Kinlough-Rathbone is a Senior Research Fellow of the Ontario Heart Foundation.

REFERENCES

1. U. S. v. Euler, in *Catecholamines* (Eds. H. Blaschko and E. Muscholl), Handbuch der experimentellen Pharmakologie, Heffter Heubner, New Series XXXIII, p. 186. Springer, Berlin (1972).
2. F. B. Hughes and B. B. Brodie, *J. Pharmac. exp. Ther.* **127**, 96 (1959).
3. A. Pletscher, *Br. J. Pharmac. Chemother.* **32**, 1 (1968).
4. A. Pletscher, M. DaPrada, K. H. Berneis and J. P. Tranzer, *Experientia* **27**, 993 (1971).
5. H.-J. Reimers, D. J. Allen, J.-P. Cazenave, I. A. Feuerstein and J. F. Mustard, *Biochem. Pharmac.* **26**, 1645 (1977).
6. H.-J. Reimers, M. A. Packham, J.-P. Cazenave and J. F. Mustard, *Biochem. Pharmac.* **26**, 1657 (1977).
7. G. Maina, *Biochim. biophys. Acta* **333**, 481 (1974).
8. N. G. Ardlie, M. A. Packham and J. F. Mustard, *Br. J. Haemat.* **19**, 7 (1970).
9. N. G. Ardlie, D. W. Perry, M. A. Packham and J. F. Mustard, *Proc. Soc. exp. Biol. Med.* **136**, 1021 (1971).
10. H. Holmsen, H. J. Day and E. Storm, *Biochim. biophys. Acta* **186**, 254 (1969).
11. R. L. Kinlough-Rathbone, A. Chahil, M. A. Packham and J. F. Mustard, *Lab. Invest.* **32**, 352 (1975).
12. D. C. B. Mills, *Nature, Lond.* **243**, 220 (1973).
13. H.-J. Reimers, M. A. Packham and J. F. Mustard, *Blood* **49**, 89 (1977).
14. F. A. McElroy, R. L. Kinlough-Rathbone, N. G. Ardlie, M. A. Packham and J. F. Mustard, *Biochim. biophys. Acta* **253**, 64 (1971).
15. Q. Z. Hussain and T. F. Newcomb, *J. appl. Physiol.* **19**, 297 (1964).
16. E. H. Mürer, *Biochim. biophys. Acta* **162**, 320 (1968).
17. J. Muenzer, E. C. Weinbach and S. M. Wolfe, *Biochim. biophys. Acta* **376**, 237 (1975).
18. M. H. Fukami, H. Holmsen and J. Bauer, *Biochim. biophys. Acta* **428**, 253 (1976).
19. H. Holmsen and H. J. Weiss, *Blood* **39**, 197 (1972).
20. H. Holmsen, in *Clinics in Hematology* (Ed. J. R. O'Brien), Vol. 1, No. 2, p. 235. W. B. Saunders Co. Ltd., London (1972).
21. H. Holmsen, C. A. Setkowsky and H. J. Day, *Biochem. J.* **144**, 385 (1974).
22. B. Century and M. K. Horwitt, *Proc. Soc. exp. Biol. Med.* **91**, 493 (1956).
23. G. E. Rivard, J. D. McLaren and R. F. Brunst, *Biochim. biophys. Acta* **381**, 144 (1975).