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EFFECT OF RESERPINE ON ADENOSINE UPTAKE AND METABOLISM, AND SUBCELLULAR TRANSPORT OF PLATELET ADENOSINE TRIPHOSPHATE IN WASHED RABBIT PLATELETS

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Abstract—There is a slow exchange of adenine nucleotides between the metabolically active (cytoplasmic) pool and the releasable amine storage organelle pool of blood platelets. Reserpine is known to inhibit serotonin uptake into platelet storage organelles. Therefore, we have determined whether reserpine also inhibits the uptake of adenine nucleotides from the cytoplasm into the storage organelles of rabbit platelets. Transport of adenine nucleotides from the metabolically active pool into the releasable amine storage granule pool was followed by labeling the metabolically active pool of adenine nucleotides by incubating the platelets with [^{14}C]adenosine or [^{14}C]adenine. Practically complete release of amine storage granule constituents was induced at various times in aliquots of the labeled platelet suspensions by treatment with a high concentration of thrombin (0.45 units/ml). The fraction of the total labeled [^{14}C]ATP released was taken as a measure of ATP transport from the metabolically active pool into the releasable pool. Reserpine (0.2 and 2 μM) decreased the rate of ATP transport into the storage granules by about 50 per cent. Platelets obtained from rabbits that had received 5 mg/kg of reserpine intraperitoneally 18 hr prior to the collection of blood released less ATP and ADP than control platelets from animals that had not received any drugs. This was not due to inhibition of the release reaction by reserpine. Since reserpine reduces the amount of adenine nucleotides in the storage granules, we conclude that if it affects the rate of efflux of adenine nucleotides from the granules at all, this effect must be slight compared with the inhibition of the uptake into the granules. Reserpine was also found to decrease the incorporation of [8-^{14}C]adenosine into platelet adenine nucleotides by inhibiting adenosine uptake into the platelets noncompetitively ($K_i = 2\ \mu$M). Inosine uptake was also inhibited by reserpine. The effect of reserpine on adenosine uptake was reversible. In contrast, the effect of reserpine on ATP transfer from the metabolically active pool into the releasable pool was irreversible. This is in keeping with earlier observations that some reserpine binds to platelets reversibly and some binds irreversibly.

It has been shown previously that there is a slow transfer of adenine nucleotides from the metabolic pool (cytoplasm) into the releasable amine storage granule pool of both rabbit and human platelets [1, 2]. This transfer of adenine nucleotides is much slower than the transfer of serotonin from the cytoplasm into the amine storage granules of platelets [1, 3]. Reserpine is known to inhibit serotonin uptake into platelet storage granules [4]. The work of Da Prada *et al.* [5] has shown that the ATP concentration in platelet storage organelles from reserpine-treated rabbits is less than that in storage organelles of platelets obtained from normal rabbits. Therefore, it was the object of the present studies to determine whether reserpine inhibits the adenine nucleotide transport from the metabolic pool into the releasable amine storage granule pool in addition to its inhibitory effect on serotonin uptake by the storage granules. Transfer of [^{14}C]ATP from the metabolic pool into the releasable pool of platelets was examined in these experiments by using platelets in which the metabolically active ATP pool had been labeled by incubating the platelets with [8-^{14}C]adenosine or [U-^{14}C]adenine. In conjunction with these studies, we also examined whether reserpine has an effect on [8-^{14}C]adenosine uptake and/or incorporation into platelet ATP.

MATERIALS AND METHODS

Non-radioactive compounds. Bovine thrombin was obtained from Parke, Davis & Co., Detroit, MI. HEPES (*N*-2-hydroxyethyl-piperazine-*N*′-2-ethane sulfonic acid) was purchased from Sigma Chemical Co., St. Louis, MO. Pyruvate kinase and phosphoenolpyruvate were obtained from C. F. Boehringer, Mannheim, New York, NY. Luciferin–luciferase was from E. I. Dupont de Nemours & Co., Wilmington, DE. Reserpine (Serpasil) was obtained from Ciba Co. Ltd., Dorval, Quebec. Reserpine powder from two sources (Matheson, Coleman & Bell, East Rutherford, NJ, and Ciba Co. Ltd., Dorval, Quebec) was dissolved in absolute ethanol at a concentration of 0.33 mM. In all experiments *in vitro*, similar results were obtained with either Serpasil or reserpine powder dissolved in ethanol. Apyrase (EC 3.6.1.5) was prepared by a slight modification [6] of the method of Molnar and Lorand [7]. This preparation hydrolyzed 18 nmoles ATP/min/μg of protein and

300 nmoles ADP/min/μg of protein (3 mg protein/ml of apyrase).

Radioactive compounds. [8-^{14}C]adenosine (sp. act. > 50 mCi/m-mole), [U-^{14}C]adenine (sp. act. > 225 mCi/m-mole) and [^{14}C]inosine (sp. act. between 40 and 60 mCi/m-mole) were obtained from Amersham/Searle Corp. (Arlington Heights, IL).

Platelet suspensions. Suspensions of washed platelets in Tyrode solution containing 0.35% albumin were prepared from rabbit blood by the method described by Ardlie *et al.* [8]. In experiments in which the platelet suspension was kept at 37° for extended periods of time, HEPES buffer was added to the suspending medium at a final concentration of 5 mM. Apyrase (0.2 μl/ml) was added to the platelet suspension in those experiments in which neither the release of total ATP and ADP nor radioactive ATP and ADP was to be determined [9].

Incorporation of [8-^{14}C]adenosine into platelets. The initial rate of [8-^{14}C]adenosine incorporation was studied in platelets suspended at a concentration of 500,000/μl in Tyrode solution at pH 7.35 containing 0.35% albumin, 5 mM HEPES buffer and apyrase. Platelets were kept at 37° in 1-ml aliquots in conical plastic Eppendorf tubes. Then 0.01 ml of 0.154 M NaCl, 0.01 ml of absolute ethanol or 0.01 ml reserpine (0.4, 4 or 10 μM) was added and incubated for 30 sec. At zero time, 0.007 ml [8-^{14}C]adenosine (0.4 to 54 μM) was added and mixed. For final adenosine concentrations greater than 1 μM, unlabeled adenosine was included. At 30 sec, a 0.1-ml sample was removed from the incubation mixture and transferred immediately into 2 ml of Tyrode solution containing 0.35% albumin, 1 mM unlabeled adenosine and 0.4% paraformaldehyde [10] to stop immediately further [8-^{14}C]adenosine uptake. This solution was kept on melting ice. This 2.1-ml sample was then filtered and washed with 8 ml of ice-cold (0°) modified Tyrode solution (no magnesium, no calcium) on a Millipore filter HAWP, diameter 25 mm, pore size 0.45 μm (Millipore Corp., Bedford, MA) placed on a Millipore filter holder (Cat. No. 10.025.02) under vacuum. (The filters were soaked overnight before the day of use in modified Tyrode solution and transferred to a Tyrode solution containing 0.35% albumin and 1 mM adenosine 4–5 hr before the commencement of the experiment.) Filtration and washing required about 15 sec. The filters were air dried in liquid scintillation counting vials before 10 ml of a toluene-based liquid scintillation fluid [3] was added to each vial and counted in a β-scintillation counter at an efficiency of approximately 70 per cent. The counts per min obtained from the filtered samples were corrected for unspecific binding to the filter and converted into nmoles adenosine taken up per 10^9 platelets, allowing for variations in the specific radioactivity. At a concentration of 4 μM, [8-^{14}C]adenosine uptake into platelets was linear for at least 120 sec.

Metabolism of [8-^{14}C]adenosine in washed platelets. [8-^{14}C]adenosine was added to a suspension of washed platelets (10^6/μl) at an initial concentration of 16 μM. At indicated time intervals up to 60 min after the addition of [8-^{14}C]adenosine, aliquots from the platelet suspension (0.2 ml) were extracted with 0.4 ml of ice-cold perchloric acid (final concn 3%) containing unlabeled adenosine metabolites in amounts giving final concentrations of about 1.6 mM in the extract. After neutralization with 0.2 ml potassium citrate (26%) and removal of $KClO_4$ by centrifugation, 0.02-ml samples were subjected to descending paper chromatography using methods described by Randerath and Struck [11] and Cain *et al.* [12]. The positions of individual adenosine metabolites ([^{14}C]ATP, [^{14}C]ADP, [^{14}C]AMP, [^{14}C]IMP, [^{14}C]inosine and [^{14}C]hypoxanthine) were identified under ultraviolet light, and appropriate areas were cut out, placed in vials and counted by liquid scintillation spectrometry [13]. Aliquots of the neutralized extract were also counted directly. In all samples the radioactivity in the identified metabolites accounted for more than 90 per cent of the radioactivity measured in the neutralized extract directly.

In some experiments, samples of supernatant fluid obtained by centrifuging 1 ml of the platelet suspension for 1 min at 12,000 *g* were also extracted with perchloric acid, and [8-^{14}C]adenosine as well as its metabolites was separated by the methods described above. In these experiments, 16 μM [8-^{14}C]adenosine was also added to (unlabeled) platelet suspension supernatant fluid (obtained by centrifuging a sample of platelet suspension at 12,000 *g* for 2 min in an Eppendorf centrifuge 3200), and [8-^{14}C]adenosine metabolites were determined at the same time intervals that were used in the experiments with platelet suspensions. This made it possible to calculate the extent of the platelet-dependent conversion of [8-^{14}C]adenosine into its metabolites.

Transfer of [^{14}C]ATP from the non-releasable (metabolic) pool into the releasable pool of platelets. Washed rabbit platelets suspended in Tyrode solution containing 0.35% albumin were incubated with 20 μCi [^{14}C]adenosine or 40 μCi [^{14}C]adenine for 60 min at 37° to label their metabolic ATP pool [14]. Platelets were removed by centrifugation and resuspended in Tyrode solution containing 0.35% albumin and 5 mM HEPES at a platelet count of 1.0×10^6/μl. This platelet suspension was kept in a conical glass tube covered with parafilm at 37° for up to 7 hr. At hourly intervals, beginning 2 hr after the addition of [^{14}C]adenosine or [^{14}C]adenine to the platelet suspension, the release reaction was induced in 1-ml aliquots with thrombin (0.45 units/ml). Control samples were treated with Tyrode solution. The platelet suspension was kept for 3 min in a shaking device (170 cycles/min) at 37°. The suspension was then rapidly transferred to an Eppendorf centrifugation tube and the reaction was stopped by centrifuging for 1 min at 12,000 *g* in an Eppendorf microcentrifuge 3200 (Brinkmann Instruments, Rexdale, Ontario). The amounts of [^{14}C]ATP and its metabolites were determined chromatographically in aliquots of the platelet suspension, in the supernatant fluid from the platelet suspension treated with Tyrode solution, and the supernatant fluid from the platelet suspension treated with thrombin. From these data the amount of [^{14}C]ATP converted to other labeled compounds and the percentage of [^{14}C]ATP released from the platelets were calculated. In some experiments, the released ATP was determined by the firefly luminescence method [15] and the changes in the specific radioactivity of the released ATP were calculated.

Experiments in vivo. Suspensions of washed platelets were prepared from rabbits that had been given an intraperitoneal injection of 5 mg reserpine/kg 18 hr prior to the collection of blood. This dose was based on that used by Shore *et al.* [16]. Control platelet suspensions were prepared from rabbits which had not been treated with reserpine. One ml of platelet suspension ($1.0 \times 10^6/\mu l$) was warmed at 37° for 5 min and then incubated with the control solution or with thrombin solution (0.45 units/ml) for 3 min at 37° in a shaking device. Apyrase was omitted from the suspending medium. After the reaction, the suspension was rapidly transferred into an Eppendorf centrifugation tube and centrifuged for 1 min at 12,000 *g* in an Eppendorf centrifuge. Aliquots of the total platelet suspension and supernatant fluid were taken for ATP and ADP determination using the firefly luminescence method [15].

In some experiments, the metabolic ATP pool was labeled by incubating the platelets with [^{14}C]adenosine *in vitro*. Platelets were injected into rabbits that also received 5 mg reserpine/kg intraperitoneally immediately after infusion of the platelets or into control rabbits that did not receive any drugs. Platelets were reharvested from blood collected 18–20 hr after their infusion. Suspensions of washed platelets were prepared and the release of [^{14}C]ATP and its metabolites was measured as described above.

RESULTS

Effect of reserpine on [8-^{14}C]adenosine uptake and metabolism by platelets. When [8-^{14}C]adenosine (16 μM) was incubated with a suspension of washed platelets, the main metabolites formed were [^{14}C]ATP, [^{14}C]ADP, [^{14}C]inosine and [^{14}C]hypoxanthine. The amounts of [^{14}C]AMP and [^{14}C]IMP found in the platelet suspension were negligible (Table 1). While all of the [^{14}C]ATP and almost all of the [^{14}C]ADP appeared to be platelet bound, practically all of the [^{14}C]inosine and [^{14}C]hypoxanthine were recovered in the supernatant fluid obtained after centrifugation of the platelet suspension (Table 1). Much smaller amounts of [^{14}C]inosine and practically no [^{14}C]hypoxanthine were formed when the supernatant fluid removed from a platelet suspension was incubated with [^{14}C]adenosine. Reserpine (4 μM) reduced the rate of [8-^{14}C]adenosine disappearance from the platelet suspension, and diminished the rate and the total amounts of [^{14}C]ATP and [^{14}C]ADP formed within the platelets (Table 1). Similarly the initial rate of [^{14}C]inosine formation was reduced. However, at the end of the 60-min incubation period, the total amount of [^{14}C]inosine that had accumulated in the suspending fluid was greater in the presence of reserpine than in its absence. In contrast to the increased accumulation of [^{14}C]inosine, there was less accumulation of [^{14}C]hypoxanthine in the platelet-suspending fluid in the presence of reserpine (Table 1). The combined accumulation of [^{14}C]inosine and [^{14}C]hypoxanthine within 60 min of addition of [8-^{14}C]adenosine to the platelet suspension was greater in the presence of reserpine than in its absence. Although some [^{14}C]inosine was formed when [^{14}C]adenosine was added to the supernatant fluid removed from a platelet suspension, the amount that accumulated was less than in the presence of platelets, and reserpine did not affect the rate or extent of [^{14}C]inosine formation (Table 1).

The cause of the increased accumulation of [^{14}C]inosine and diminished accumulation of [^{14}C]hypoxanthine in the platelet-suspending fluid in the presence of reserpine was investigated in an experiment in which platelets suspended in Tyrode solution containing 0.35% albumin were incubated with [^{14}C]inosine in either the absence or presence of reserpine (Fig. 1). Reserpine inhibited the platelet-dependent conversion of [^{14}C]inosine to [^{14}C]hypoxanthine.

Table 2 shows that the effects of reserpine *in vitro* on [8-^{14}C]adenosine metabolism in platelets were partially reversed by resuspending them in fresh Tyrode solution containing 0.35% albumin before adding [8-^{14}C]adenosine. When platelets were isolated from the blood of rabbits that had received 5 mg reserpine/kg 18 hr prior to the collection of blood, the metabolic conversion of [8-^{14}C]adenosine by platelets was the same as by platelets obtained from the blood of untreated rabbits (data not shown).

The rapid metabolism of [8-^{14}C]adenosine in blood platelets and its partial conversion to [^{14}C]inosine and [^{14}C]hypoxanthine, which are not retained in the platelets, pose problems for the determination of [8-^{14}C]adenosine uptake by platelets. To minimize these problems, adenosine uptake was measured after short incubation times (0.5 min) of the platelet suspension with μM concentrations of [8-^{14}C]adenosine. Figure 2 shows that adenosine uptake was linear for at least 2 min when platelets were incubated with [8-^{14}C]adenosine at a concentration of 4 μM. In a double reciprocal plot of the velocities of [8-^{14}C]-adenosine uptake vs adenosine concentration, a K_m of 10 μM for [8-^{14}C]adenosine uptake was obtained (Fig. 3). An Eadie–Hofstee plot of the same data gave a maximum velocity of adenosine uptake of 0.540 nmole/0.5 min/10^9 platelets (Fig. 3). Reserpine inhibited adenosine uptake. This inhibition appeared to be noncompetitive (Fig. 3) with a K_i of 2 μM as obtained from a Dixon plot.

Effect of reserpine on transfer of metabolic ATP into the releasable compartment. Platelets from rabbits that had been treated with reserpine released less ATP and ADP upon exposure to 0.45 units/ml of thrombin than the control platelets (Table 3). In normal platelets, adenine nucleotides exchange between the metabolically active pool and the releasable pool [1, 2]. Therefore, we examined whether the reduced amounts of releasable ATP and ADP in platelets from reserpine-treated animals were due to (1) impaired transfer of ATP from the cytoplasmic, metabolically active compartment into the storage organelles, or (2) inhibition of the platelet release reaction. To investigate these possibilities, the transfer of metabolically active ATP (labeled with [8-^{14}C]adenosine or [U-^{14}C]-adenine) into the releasable pool was followed in the presence or absence of reserpine. Because of the effect of reserpine *in vitro* on adenosine uptake, platelets were labeled with [8-^{14}C]adenosine or [U-^{14}C]-adenine prior to incubation with this drug. After incubation for 1 hr with the labeled compounds, the platelet suspension was divided into two parts: reser-

pine was added to one part, the other part served as a control. At hourly intervals up to 7 hr, the amount of [^{14}C]ATP that could be released from the platelets was determined by inducing the release reaction with a high concentration of thrombin (0.45 units/ml) in aliquots of the two suspensions. Seven hr after the platelets were labeled, about 24 per cent of the total platelet [^{14}C]ATP could be released from the control platelets but only 14 per cent could be released from the platelets that had been treated with reserpine (Fig. 4). Reserpine had the same effect at concentrations of 0.2 or 2 μM. Washing the platelets in calcium-free Tyrode solution did not remove the effect of reserpine.

Next, an experiment was done in which the specific radioactivity of the released [^{14}C]ATP was deter-

Table 1. Effect of reserpine on [8-^{14}C]adenosine metabolism by washed platelets*

Metabolite	Inhibitor	Incubation medium†	Time after addition of [8-^{14}C]adenosine (min)									
			1	5	10	15	20	25	30	40	50	60
[^{14}C]adenosine	Tyrode	P_I	87	64	40	24	13	7	5	2	1	1
		P_S	93	68	43	27	16	9	5	2	1	0
		S	100	99	100	98	98	95	86	82	85	77
	Reserpine	P_I	84	84	71	62	50	48	42	29	20	12
		P_S	96	86	77	67	56	49	42	28	19	12
		S	103	99	97	94	92	96	94	91	90	88
[^{14}C]ATP	Tyrode	P_T	5	13	21	28	33	37	37	40	41	40
		P_S	0	0	0	0	0	0	0	0	0	0
		S	0	0	0	0	0	0	0	0	0	0
	Reserpine	P_T	1	3	6	9	12	14	17	21	24	25
		P_S	0	0	0	0	0	0	0	0	0	0
		S	0	0	0	0	0	0	0	0	0	0
[^{14}C]ADP	Tyrode	P_T	0	1	2	5	5	5	6	8	10	11
		P_S	0	0	0	0	0	1	1	1	2	3
		S	0	0	0	0	0	0	0	0	0	0
	Reserpine	P_T	0	0	0	1	2	2	2	4	4	4
		P_S	0	0	0	0	0	0	0	0	0	0
		S	0	0	0	0	0	0	0	0	0	0
[^{14}C]AMP	Tyrode	P_T	0	0	0	0	0	0	0	0	1	1
		P_S	0	0	0	0	0	0	0	0	0	0
		S	0	0	0	0	0	0	0	0	0	0
	Reserpine	P_T	0	0	0	0	0	0	0	0	0	0
		P_S	0	0	0	0	0	0	0	0	0	0
		S	0	0	0	0	0	0	0	0	0	0
[^{14}C]IMP	Tyrode	P_T	3	4	3	3	1	2	2	1	2	1
		P_S	0	0	0	0	0	0	0	0	0	0
		S	0	0	0	0	0	0	0	0	0	0
	Reserpine	P_T	0	0	0	1	1	1	1	1	1	0
		P_S	0	0	0	0	0	0	0	0	0	0
		S	0	0	0	0	0	0	0	0	0	0
[^{14}C]inosine	Tyrode	P_T	7	17	25	29	30	31	29	26	25	23
		P_S	6	17	25	30	30	29	28	25	24	22
		S	1	2	4	5	6	7	8	11	13	15
	Reserpine	P_T	3	8	15	21	29	34	36	46	47	47
		P_S	4	9	16	23	27	32	38	45	43	48
		S	1	2	4	5	6	7	8	11	12	15
[^{14}C]hypoxanthine	Tyrode	P_T	1	4	10	14	17	20	23	25	29	30
		P_S	1	4	9	14	16	20	22	25	28	29
		S	0	0	0	0	1	1	1	1	1	1
	Reserpine	P_T	0	1	1	1	2	2	3	5	8	12
		P_S	6	1	1	1	2	2	3	5	8	11
		S	0	0	0	0	0	0	0	0	1	1
Recovery‡	Tyrode	P_T	103	103	101	103	99	102	102	102	109	107
		P_S	102	102	102	100	101	102	98	101	101	102
		S	101	101	104	103	105	103	95	94	99	93
	Reserpine	P_T	88	96	93	95	96	101	101	104	104	100
		P_S	99	98	99	106	100	101	103	101	94	99
		S	104	101	101	99	98	103	102	102	103	104

* The conditions of the experiment are as follows: 16 μM [^{14}C]adenosine (initial concn); platelet count: $1.0 \times 10^6/\mu$l; reserpine 4 μM. This was one of three similar experiments.

† P_T: metabolite was extracted with perchloric acid from total platelet suspension, to which [8-^{14}C]adenosine had been added. P_S: metabolite was extracted with perchloric acid from supernatant fluid obtained by centrifugation from platelet suspension that had been incubated with [8-^{14}C]adenosine. S: metabolite was extracted with perchloric acid from platelet-suspending fluid that had been incubated with [8-^{14}C]adenosine after its separation from the platelets by centrifugation.

‡ Percentage of the radioactivity accounted for.

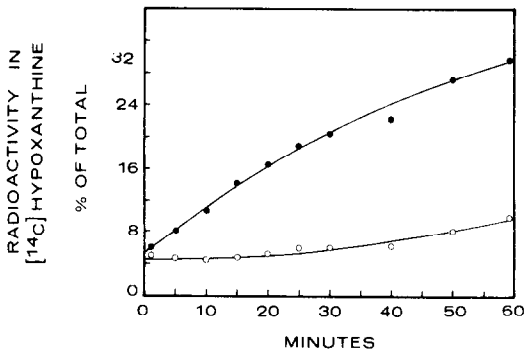

Fig. 1. Effect of reserpine (4 μM) on the conversion by washed rabbit platelets of added [^{14}C]inosine (initial conc 16 μM) to [^{14}C]hypoxanthine. The platelet concentration was 1.0 × $10^6/\mu$l. There was no appreciable conversion of [^{14}C]inosine to [^{14}C]hypoxanthine in the absence of platelets. Key: reserpine (O——O), control (●——●).

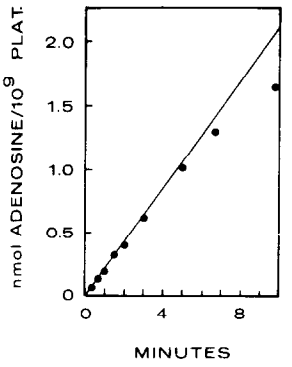

Fig. 2. Effect of time on the accumulation of radioactivity by washed rabbit platelets upon incubation with [8-^{14}C]-adenosine (initial concn 4 μM). The platelet concentration was 0.5 × $10^6/\mu$l. Accumulation of radioactivity in the platelets was measured by the filtration method described in Materials and Methods. The initial rate of accumulation of radioactivity was taken as a measure for the adenosine uptake by platelets (0.206 nmole/min/10^9 platelets). A practically identical result was obtained when the platelet count was adjusted to 1.0 × $10^6/\mu$l (0.199 nmole/min/10^9 platelets).

mined since, if a reduced rate of transfer of [^{14}C]ATP into the granules were responsible for the reduced release, the specific radioactivity of the released [^{14}C]ATP would be less in the presence of reserpine. Figure 5 shows that, in the presence of 2 μM reserpine, the specific radioactivity of the releasable [^{14}C]ATP increased more slowly than it did in the absence of reserpine.

To study the transfer over a longer period of time, experiments were done in which the labeled platelets were infused into rabbits and reharvested after 18 hr. The platelets that had been treated with reserpine before their infusion into rabbits released less of their radioactive [^{14}C]ATP (41 ± 2 per cent) upon addition of thrombin (0.45 units/ml) than the control platelets (53 ± 3 per cent) that had not been treated with reserpine (means ± S.E.M.; N = 6; P < 0.05). When reserpine-treated platelets were injected into rabbits that also received 5 mg reserpine/kg, immedi-

Table 2. Effect of reserpine on metabolic conversion of [8-^{14}C]adenosine by suspensions of washed rabbit platelets*

Metabolite	Type of platelet suspension	Time after addition of [^{14}C]adenosine (min)						
		1	10	20	30	40	50	60
[^{14}C]adenosine	A	87	53	29	16	8	4	2
	B	98	90	76	59	48	37	32
	C	97	72	61	47	34	22	18
[^{14}C]ATP	A	5	16	29	40	40	41	39
	B	1	4	7	9	11	15	16
	C	1	8	17	23	27	30	35
[^{14}C]ADP	A	2	2	3	5	5	7	5
	B	1	1	1	2	1	2	2
	C	1	2	2	4	5	3	7
[^{14}C]inosine	A	5	21	27	31	31	28	27
	B	3	14	25	32	41	44	50
	C	4	13	22	31	33	32	39
[^{14}C]hypoxanthine	A	3	8	12	16	18	21	21
	B	1	1	1	2	2	2	2
	C	1	1	2	4	5	6	10

* In these experiments, [^{14}C]adenosine (initial concn 16 μM) was added to a suspension of washed rabbit platelets (platelet count $10^6/\mu$l) in the absence (A) or presence (B) of 4 μM reserpine. Suspension C had been treated with reserpine (4 μM) and was then resuspended in Tyrode solution containing 0.35% albumin before addition of [^{14}C]adenosine. Aliquots of the suspensions were extracted with 3% perchloric acid at the indicated times, and [^{14}C]ATP, [^{14}C]ADP, [^{14}C]AMP, [^{14}C]IMP, [^{14}C]hypoxanthine and [^{14}C]inosine and [^{14}C]adenosine were separated chromatographically. The radioactivity in [^{14}C]AMP and [^{14}C]IMP was minimal and has been omitted from this table. In similar experiments it was found that [^{14}C]ATP and [^{14}C]ADP were almost completely platelet bound, whereas [^{14}C]adenosine, [^{14}C]inosine and [^{14}C]hypoxanthine were almost exclusively in the platelet-suspending fluid (see Table 1). The values given in this table are expressed as a percentage of the total radioactivity added.

Table 3. Effect of reserpine on releasable ATP + ADP of rabbit platelets*

	ATP + ADP (μmoles/10^{11} platelets)		Significance of difference between means
	Control platelets	Reserpine-treated platelets†	
Platelet ATP + ADP content	6.6 ± 0.7	5.8 ± 0.5	$P < 0.2$
Released ATP + ADP‡	4.1 ± 0.5	3.0 ± 0.4	$P < 0.05$
Per cent of total ATP + ADP released	62	52	

* Suspensions of washed platelets were prepared from blood obtained from either the reserpine-treated animals or the control animals that had not received any reserpine. Results represent means ± S. E. of five experiments.

† Reserpine (5 mg/kg) was injected intraperitoneally into rabbits 18 hr prior to collection of the blood.

‡ The release reaction was induced with 0.45 units/ml of thrombin, a concentration known to cause complete release of amine storage granule contents from control platelets.

ately after infusion of the platelets, release of [^{14}C]ATP was less than from platelets that had only been treated with reserpine *in vitro* (Table 4).

DISCUSSION

The results reported demonstrate two cellular effects of reserpine that have not been described so far to the best of our knowledge: (1) reserpine inhibits the incorporation of adenosine into the metabolic pool of adenosine triphosphate (ATP), and (2) it inhibits the transfer of ATP from the metabolic pool into the releasable granule pool of blood platelets.

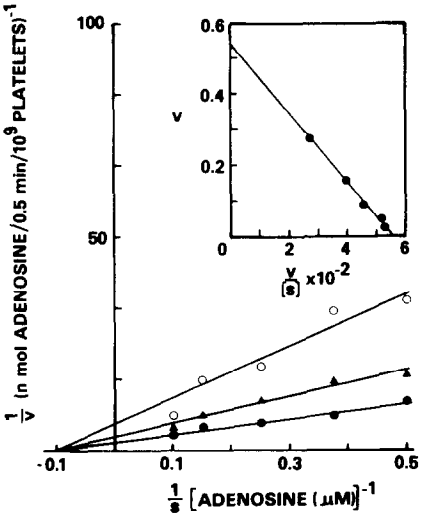

Fig. 3. Kinetic analysis of the uptake of adenosine by washed rabbit platelets and its inhibition by reserpine. Uptake of adenosine in the absence (●——●) or presence of two different reserpine concentrations (▲——▲, 4 μM reserpine; ○——○, 10 μM reserpine) was measured by the filtration method described in Materials and Methods. The reciprocals of the velocities of adenosine uptake are plotted against the reciprocal of the adenosine concentration.

The insert shows an Eadie–Hofstee plot of the velocities of adenosine uptake at different adenosine concentrations in the absence of reserpine. v = initial velocity (nmoles/0.5 min/10^9 platelets), s = concentration of adenosine (μM). All points represent the means of triplicate determinations. This was one of three similar experiments.

Effect of reserpine on uptake of [8-^{14}C]*adenosine by platelets and its metabolism within the platelets.* The incorporation of radioactively labeled adenosine into platelet adenine nucleotides has been studied extensively [18–23]. Holmsen and Rozenberg [19] have provided evidence that the incorporation of adenosine into platelet adenine nucleotides depends on rapid phosphorylation by adenosine kinase of adenosine transported across the platelet membrane. Our finding that any [8-^{14}C]adenosine remaining was in the supernatant fluid of platelet suspensions incubated with this compound, is consistent with the suggestion of Holmsen and Rozenberg [19] that there is no free intracellular adenosine provided μM concentrations of adenosine are used. However, the results from the present study show that adenosine can also be deaminated to inosine by the platelets and accumulate subsequently in the extracellular fluid. This is in contrast to the conclusion by Holmsen and Rozenberg [19] that adenosine seems to be exposed only to adenosine kinase and not to adenosine deaminase upon entry into human platelets, but is consistent

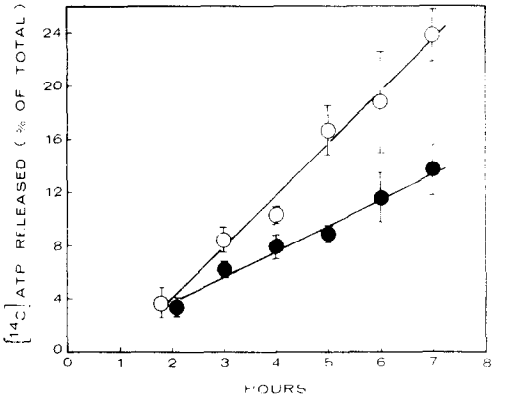

Fig. 4. Effect of reserpine (2 μM) on time-dependent increase of [^{14}C]ATP that could be released from washed rabbit platelets prelabeled with [^{14}C]adenosine. The detailed labeling procedure is described in Materials and Methods. At the times indicated, aliquots of the platelet suspension (1 × 10^6/μl) were removed, and release was induced with 0.45 units/ml of thrombin. Means and S.E.M. of five experiments. Similar results were observed with 0.2 μM reserpine. Key: reserpine (●——●), control (○——○).

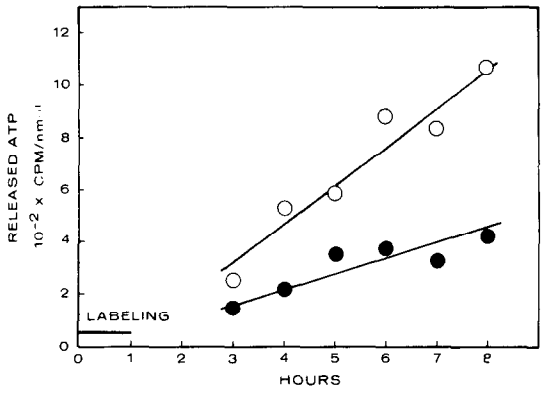

Fig. 5. Effect of reserpine (2 μM) on the time-dependent increase in specific radioactivity of ATP released from washed rabbit platelets prelabeled with [^{14}C]adenosine. Details of the 1-hr labeling procedure (black bar) are described in Materials and Methods. At the times indicated, aliquots of the platelet suspension were removed, and the release reaction was induced with 0.45 units/ml of thrombin. Aliquots of the supernatant were taken for ATP determination with the luciferin–luciferase method and for chromatographic separation of radioactive adenine nucleotides in perchloric acid extracts. Values were corrected for total ATP and radioactive ATP in the supernatant fluid before stimulation with thrombin. For details see Materials and Methods. Similar results were obtained when platelets were prelabeled with [^{14}C]adenine. This was one of two similar experiments. Key: reserpine (●——●), control (○——○).

with more recent findings by Sixma *et al.* [24] and the earlier observations of Ireland and Mills [18] in suspensions of washed human platelets that "platelets as well as accumulating phosphorylated derivatives of adenosine convert adenosine into inosine (and convert inosine into hypoxanthine)". It is possible that the deamination of adenosine by platelets has been missed in experiments employing platelet-rich plasma [18, 19] because of the high adenosine deaminase activity in plasma [22].

Deamination of adenosine as the first metabolic step after its entry into the cell has also been described in other cells such as polymorphonuclear leukocytes [25]. For red cells it has been proposed that the adenosine concentration and its affinity for adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4.) and adenosinekinase (ATP: adenosine-5'-phosphotransferase, EC 2.7.1.20) determine whether the adenosine will be phosphorylated or deaminated [26]. Sixma *et al.* [24] have recently put forward an alternative hypothesis. They suggested that adenosine can be taken up by platelets by two different carrier-mediated transport systems. These authors provided some evidence that adenosine at low (μM) concentrations is taken up preferentially via a low K_m system and directly incorporated into adenine nucleotides. In contrast, adenosine taken up by a high K_m system (mM adenosine concentrations) is converted to inosine and hypoxanthine or incorporated into adenine nucleotides. In the present experiments, only low concentrations of adenosine were used (μM range) and short incubation times were chosen (0.5 min) for the study of adenosine uptake, thus minimizing the problems arising from intracellular deamination of [8-^{14}C]adenosine. (Deamination of adenosine and subsequent rapid loss of inosine from the platelets into the extracellular fluid may lead to underestimation of adenosine uptake.) Under these conditions a Michaelis–Menten constant of 10 μM for adenosine uptake by washed rabbit platelets was found. This value is very similar to the value of 9.8 μM reported by Sixma *et al.* [24] for the low K_m system of adenosine uptake by human platelets. Similar results have also been reported for adenosine uptake by rabbit polymorphonuclear leukocytes (10 μM) [25] and for canine heart (11.6 μM) [27].

There is some kinetic evidence that the entry and metabolism of adenosine are separate events [25, 28, 29]. Therefore, inhibitors could interfere with either the carrier-mediated uptake across the mem-

Table 4. Effect of reserpine on the transfer of [^{14}C]ATP from the non-releasable, metabolic pool into the releasable pool of rabbit platelets*

	Amount transferred (cpm/10^9 platelets)			
	Control platelets		Reserpine-treated platelets	
	[^{14}C]ATP	[^{14}C]ADP	[^{14}C]ATP	[^{14}C]ADP
Exp. 1				
Platelet	52,500	4,900	46,600	7,000
Released with 0.45 units/ml thrombin	25,300	4,800	11,600	2,900
Exp. 2				
Platelet	26,300	3,400	38,800	4,600
Released with 0.45 units/ml thrombin	16,500	1,900	12,500	2,000

* Platelets labeled with [^{14}C]adenosine were treated *in vitro* with reserpine (2 μM) and injected into rabbits treated with 5 mg/kg of reserpine intraperitoneally. Platelets were reharvested from blood collected 18 hr after their injection. They were suspended in Tyrode solution containing 0.35% albumin, and the release reaction was induced with 0.45 units/ml of thrombin. The platelet count was $10^6/\mu$l. Control platelets were prepared from the blood of rabbits that had been injected with [^{14}C]adenosine-labeled platelets. These platelets had not been incubated with reserpine *in vitro* nor had the rabbits been treated with reserpine. In each experiment the values represent the mean of two observations. It is likely that the release of [^{14}C]ADP is overestimated since there is some breakdown of [^{14}C]ATP after its release into the suspending medium [17].

brane (transport step) or with the first metabolism step. The present results are compatible with the hypothesis that reserpine affected both steps. (1) It diminished uptake of adenosine by platelets since the disappearance of adenosine from the platelet-suspending fluid was slowed, and (2) reserpine inhibited the incorporation of adenosine into platelet adenine nucleotides. The inhibition of incorporation of [8-^{14}C]-adenosine into adenine nucleotides was not solely due to inhibition of adenosine uptake since the total platelet-dependent inosine (+ hypoxanthine) formation from [8-^{14}C]adenosine within 60 min was increased in the presence of reserpine. This indicates that a larger fraction of the [8-^{14}C]adenosine that entered the platelets was deaminated to inosine in the presence of reserpine. Simultaneous inhibition of adenosine uptake and the adenosine kinase reaction by inhibitors of adenosine uptake has been postulated in other cell systems as well [30].

Another possible interpretation of the effect of reserpine on adenosine metabolism is that reserpine inhibits both the low K_m and the high K_m carrier systems for adenosine uptake described by Sixma *et al.* [24]. Inhibition of the low K_m system would explain the diminished incorporation of [8-^{14}C]-adenosine into platelet adenine nucleotides in the presence of reserpine. Inhibition of the high K_m system by reserpine would account for the reduced rate of inosine formation. The increased total platelet-dependent inosine + hypoxanthine formation from [8-^{14}C]adenosine in the presence of reserpine would have to be attributed to a relatively greater effect of reserpine on the low K_m system than on the high K_m system.

Both hypotheses for the nature of the reserpine-induced inhibition of adenosine uptake by platelets assume that reserpine inhibits the low K_m carrier-mediated transport. Kinetic analysis at low adenosine concentrations (0.4 to 10 μM adenosine) showed that the reserpine-induced inhibition is noncompetitive. No further attempt was made in the present experiments to determine if the second effect of reserpine on the metabolism of adenosine described above represents an inhibition of the high K_m system [24] or an inhibition of platelet adenosine kinase.

Reserpine also diminished the platelet-dependent conversion of exogenous [^{14}C]inosine to [^{14}C]hypoxanthine. This indicates that reserpine probably also blocks membrane transport of inosine. Inhibition of inosine transport by other inhibitors of adenosine transport such as *p*-nitrobenzylthioguanosine has been shown previously [23, 31]. However, it is presently unknown whether inosine and adenosine use the same carrier-mediated transport system.

Effect of reserpine on transfer of metabolic ATP into the releasable pool of platelets. It was previously demonstrated with both rabbit and human platelets that ATP is transported from the metabolically active, non-releasable pool into the releasable storage organelle pool [1, 2]. The results of the present experiments demonstrate that in the presence of reserpine both the absolute amounts of releasable radioactive ATP and the specific radioactivity of the released [^{14}C]ATP were decreased. This must be due to inhibition of transfer of [^{14}C]ATP from the metabolic pool into the releasable granule pool in the presence of reserpine. Our conclusion that the diminished release of [^{14}C]ATP from platelets is not due to an inhibition of the thrombin-induced release reaction is supported by our previous observations [32]. In those experiments it was shown that reserpine *in vitro* did not reduce the thrombin-induced release of serotonin and adenine nucleotides. Thus, reserpine not only inhibits the transfer of serotonin across the platelet amine storage organelle membrane [4] but also inhibits the mechanism by which adenine nucleotides are transported across this granule membrane. However, the maximum transport rate of serotonin across the granule membrane is much greater than that of ATP [1, 3] so that these two processes are not likely to be linked to each other. The amount of releasable ATP + ADP in platelets from rabbits that had received reserpine 18 hr prior to blood collection was diminished. This finding is compatible with the observation of Da Prada *et al.* [5] that the ATP content of isolated storage granules from rabbits treated with reserpine is lower than that of granules of platelets from untreated rabbits. Since reserpine reduces the transport of ATP from the cytoplasm into the releasable pool, this must mean that the transport of ATP or its metabolites from the releasable pool back into the cytoplasm is decreased much less (if at all), resulting in a decreased amount of releasable storage granule ATP + ADP.

The results of the present studies may also be relevant to the earlier observation that reserpine reduces the catecholamine and nucleotide content of the (denervated) adrenal medulla [33, 34] as well as of isolated medullary granules [35].

The effect of reserpine on adenine nucleotide transport across the storage granule membrane appears to be irreversible since the inhibition of the transport of ATP from the metabolic pool to the releasable pool was sustained when platelets were washed after treatment with reserpine *in vitro* or when reserpine-treated platelets were infused into the circulation of untreated rabbits. This effect of reserpine on the transport of adenine nucleotides across the storage granule membrane is different from its effect on the uptake of adenosine across the platelet membrane. Inhibition of adenosine uptake was diminished when platelets were resuspended in fresh medium after incubation with reserpine or abolished when adenosine uptake was determined in platelets prepared from rabbits that had received reserpine 18 hr prior to the collection of blood. Thus, the effect of reserpine on adenosine uptake is reversible.

The present observations that reserpine can alter adenosine transport across the platelet plasma membrane reversibly and ATP transport (in addition to serotonin transport) across the storage granule membrane irreversibly are in keeping with the demonstration by Enna *et al.* [36] that although part of the reserpine binding to platelets is reversible, reserpine binding to the storage granule membrane is irreversible. They are also in keeping with the suggestion of Minter and Crawford [37] that both the surface membrane of platelets and the granule membranes are targets for reserpine.

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