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MOVEMENT OF SODIUM INTO HUMAN PLATELETS INDUCED BY ADP

H. FEINBERG *, W.C. SANDLER, M. SCORER, G.C. LE BRETON, B. GROSSMAN and G.V.R. BORN [a] **

Department of Pharmacology, University of Illinois College of Medicine, Chicago, Ill. (U.S.A.) and [a] Department of Pharmacology, Royal College of Surgeons of England, London (U.K.)

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Summary

1. In normal human platelets the concentrations of Na^+ and K^+ were 42.1 ± 4.3 and 98.8 ± 3.7 mequiv/l of platelet water respectively (mean ±S.E. of 22 samples).

2. When platelet-rich plasma was incubated with $^{22}Na^+$ at 37°C for 2—3 h an increase in platelet Na^+ concentration was found which was significant after 210 min. Platelet K^+ concentration did not change significantly. The platelet $^{22}Na^+$ radioactivity increased faster than did the total Na^+, suggesting a Na^+_o-Na^+ exchange process in unactivated platelets.

3. Addition of ADP to platelet-rich plasma resulted in platelet aggregation and a rapid rise (within seconds) in $^{22}Na^+$-radioactivity within the platelets and after 300 s this increase diminished toward control levels.

4. Under the same experimental conditions, ADP did not bring about an increase of $^{36}Cl^-$ in the platelets.

5. Ouabain (10^{-6} M) added to platelet-rich plasma induced an increase in Na^+ concentration and $^{22}Na^+$ radioactivity in the platelets, as well as a decrease in K^+ concentration. ADP produced a further increase in $^{22}Na^+$, which did not return toward control values, in the presence of ouabain.

6. The association of an increase in $^{22}Na^+$ but not of $^{36}Cl^-$ accompanying aggregation by ADP suggests a selective mechanism for the movement of Na^+ into platelets rather than a movement of NaCl together with water under an osmotic gradient.

* To whom correspondence should be addressed at Department of Pharmacology, University of Illinois, 835 South Wolcott Avenue, Chicago, Ill. 60612, U.S.A.

** Present address: Department of Pharmacology, University of Cambridge, U.K.

Introduction

Adenosine diphosphate (ADP) induces change in shape, aggregation and secretion in blood platelets [1—3]. As there is evidence that ADP does not cross the platelet membrane but becomes bound to it [4,5], a transduction mechanism must exist whereby events at the platelet surface initiate the morphological changes. Platelets, like other cells, maintain lower Na^+ and higher K^+ concentrations than those in their environment [6—8]. Platelets contain an Mg^{2+}-dependent (Na^+ + K^+)-stimulated ATPase [9] which is inhibited by ouabain as well as by ADP [10]. Thus, the activation of platelets by ADP (and perhaps also by other agents) may involve changes in ion flux across the cell membrane. In this paper we report that platelet activation by ADP is associated with an increased influx of Na^+.

Methods

Human platelet-rich plasma was prepared as previously described [11] by centrifuging citrated blood from apparently healthy donors at 164 × *g* for 15 min. The plasma was incubated at 37°C with $^{22}NaCl$ (0.4 μCi/μg) and with ^{125}I-labelled human serum albumin (0.01 μCi of 2.5 μCi/mg). The isotopes were obtained from the Radiochemical Centre, Amersham/Searle. Platelets from 1 ml of platelet-rich plasma were sedimented through silicone oil [12] by centrifugation in a Fisher (Model 59) centrifuge at 7000 × *g* for 1 min. This avoided preparative artifacts in ion fluxes produced by successive resuspensions of platelets in media other than plasma. Platelet pellets and samples of platelet-free plasma were prepared for radioactivity determinations as already described [12], and $^{22}Na^+$ and ^{125}I radioactivities were simultaneously measured in a Nuclear Chicago gamma counter. Samples were counted for 10 min. Settings were determined that gave negligible crossover of ^{125}I to the $^{22}Na^+$ channel (0.25 ± 0.08%) and minimal crossover of $^{22}Na^+$ to the ^{125}I channel (4.35 ± 0.007%). The small volumes of plasma trapped with the sedimented platelets were estimated from the ^{125}I present in the pellets.

The platelet $^{22}Na^+$ radioactivity was determined as the difference between total pellet $^{22}Na^+$ and $^{22}Na^+$ in the trapped plasma. Pellet $^{22}Na^+$ and ^{125}I radioactivity averaged 682 ± 28 cpm and 249 ± 22 cpm respectively (n = 67). The platelet $^{22}Na^+$ was expressed as a volume or 'space' calculated as the ratio of $^{22}Na^+$ in the platelet and $^{22}Na^+$ present in 1.0 μl of platelet-free plasma. Since the volume of trapped plasma was determined in each sample, the $^{22}N^+$ radioactivity attributable to trapped plasma could be accurately measured. The fractional crossover of $^{22}Na^+$ radioactivity into the ^{125}I channel was used to correct all ^{125}I counts; however, correction for fractional crossover did not appreciably influence the calculated ^{22}Na 'space' (e.g., 0.100 ± 0.003 μl before and 0.103 ± 0.003 μl after correction in a typical set of 4 replicates.) The amount of Na^+ presumed to have entered the platelet (in mequiv/l of platelet water) was calculated as the ratio between $^{22}Na^+$ in 10^8 platelets (shown by Feinberg et al. [12] to contain about 1.0 μl of water) and the specific activity of $^{22}Na^+$ in platelet-free plasma.

The Na^+ and K^+ in platelets was determined by flame photometry (Baird-Atomic) on a protein-free supernatant obtained by dispersing the platelet pellet in water and adding Li^+ standard and trichloroacetic acid to final concentrations of 250 ppm and 5% respectively. The amounts of Na^+ and K^+ in excess of that in the trapped plasma were assumed to be in the platelets. Platelet aggregation was measured photometrically [3] and platelets were counted by phase contrast microscopy; the counts ranged from $2-4 \cdot 10^8$ platelets per ml.

Results

In normal human platelets the Na^+ and K^+ concentrations were 42.1 ± 4.3 and 98.8 ± 3.7 mequiv/l of platelet water (mean ± S.E. of 22 samples) respectively. These values are similar to those reported by others [6,13–15]. On the other hand, platelets washed free of plasma with isotonic Tris/choline solution have been shown to contain less Na^+ and K^+ [7], whereas platelets washed with Tris/Tyrode's solution contain less K^+ and more Na^+ [8]. The values obtained with washed platelets may have been influences by ion exchanges during the washing procedure.

Low platelet Na^+ relative to plasma could be maintained by pumping out Na^+ which diffuses down a plasma-platelet concentration gradient, or by low permeability of the platelet membrane to Na^+. The platelet $^{22}Na^+$ radioactivity (expressed in μl as a $^{22}Na^+$ 'space') was measured at intervals after the addition of $^{22}NaCl$ to platelet-rich plasma (Fig. 1). The platelet $^{22}Na^+$ 'space' increased within the first 60 min and showed no significant increase during the next 150

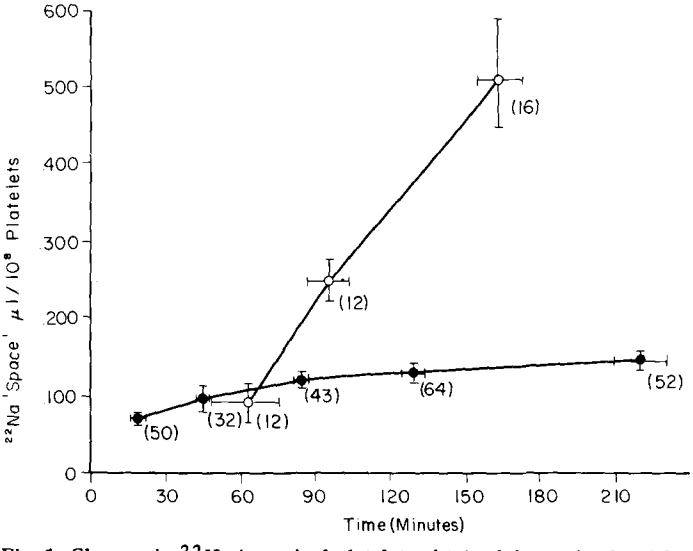

Fig. 1. Changes in ^{22}Na 'space' of platelets obtained from platelet-rich plasma incubated at 37°C in the absence (•) or presence (○) of ouabain ($1 \cdot 10^{-6}$ M). ^{22}Na 'spaces' were calculated as described under Methods and expressed as μl per 10^8 platelets. Samples were taken at slightly different times in 16 experiments; the time ranges are shown by the horizontal bars. Number of samples at each sampling time is shown in brackets. Vertical bars indicate standard errors of the means. The first sample from the plasma containing ouabain was taken 2 min after its addition.

min. If ouabain (10^{-6} M) was added to the plasma the $^{22}Na^+$ 'space' increased at a rapid rate (Fig. 1), an indication that $^{22}Na^+$ entered the platelet and equilibrated with a part or all of the platelet Na^+ available to the ouabain-sensitive efflux mechanism. In four experiments the platelet Na^+ and K^+ concentration and $^{22}Na^+$ 'space' were measured at intervals on the same samples. A significant increase in $^{22}Na^+$ occurred between 40 and 120 min and the upward trend continued over the next 80 min. The platelet Na^+ concentration increased slowly during the same period; however, only the measurement at 210 min showed a significant increase in Na^+ concentration over the initial value (Fig. 2A). No significant change occurred in platelet K^+ concentration. The apparent uptake of Na^+, based on $^{22}Na^+$ radioactivity and the specific activity of $^{22}Na^+$ in plasma, amounted to 13.6 ± 4.0% of total platelet Na^+ after 40 min and 34.4 ± 0.9% after 210 min. Thus, the increase in $^{22}Na^+$ radioactivity was faster than the increase in total Na^+, indicating an Na_o^+—Na_i^+ exchange in unactivated platelets. In the presence of ouabain both $^{22}Na^+$ 'space' and platelet Na^+ concentration increased much more than in the untreated platelet, while platelet K^+ decreased (Fig. 2B). Therefore, inhibition of the cells, Mg^{2+}-dependent ($Na^+ + K^+$)-stimulated ATPase resulted in an almost complete exchange of platelet K^+ for Na^+.

When ADP was added to platelet-rich plasma containing $^{22}NaCl$ and ^{125}I-albumin, aggregation of the platelets was accompanied by a decrease in trapped

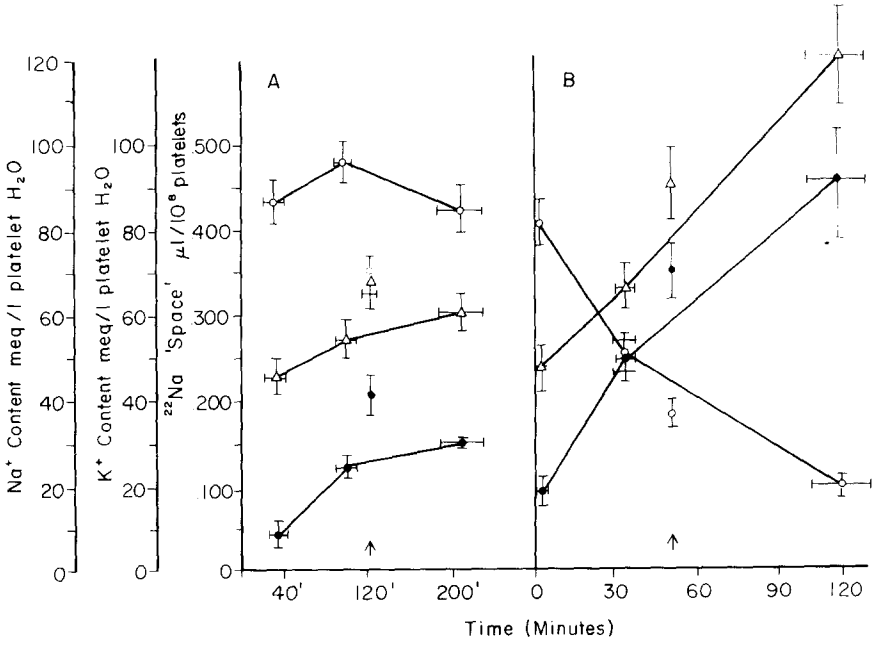

Fig. 2. Effect of ADP (10^{-5} M) on platelet Na^+ and K^+ and $^{22}Na^+$ 'space'. Platelet-rich plasma was incubated at 37°C in the absence (panel A) or presence (panel B) of ouabain (10^{-6} M). The arrow indicates the time of addition of ADP to an aliquot of the sample. Platelet $^{22}Na^+$ 'space' (●), Na^+ content (△) and K content (○) of the unstimulated platelets are shown in the lower, middle and upper curves respectively. The separate symbols aligned with the arrow depict the same measurements obtained 60 s after the addition of ADP. Samples were taken at slightly different times in 4 experiments; the time ranges are shown by the horizontal bars. Vertical bars indicate the standard error of the means.

TABLE I

EFFECT OF ADP ON PLATELET ^{22}Na "SPACE"

The ^{22}Na 'space' was calculated from measurements of plasma and pellet ^{22}Na and ^{125}I-albumin radioactivities as follows:

Pellet ^{22}Na 'space': Trapped plasma: Platelet ^{22}Na 'space':

$$\left[\left(\frac{\text{Pellet } ^{22}\text{Na cpm}}{\left(\frac{\text{Plasma } ^{22}\text{Na cpm}}{\mu\text{l of plasma}}\right)\left(\frac{\text{platelet}}{\text{count}}\right)}\right)-\left(\frac{\text{Pellet } ^{125}\text{I cpm}}{\left(\frac{\text{Plasma } ^{125}\text{I cpm}}{\mu\text{l of plasma}}\right)\left(\frac{\text{platelet}}{\text{count}}\right)}\right)\right] = \mu l/10^8 \text{ platelets}$$

Samples were taken at increasing times after the addition of ADP (10^{-5} M) to platelet-rich plasma.

		Pellet ^{22}Na 'space'	Trapped plasma $\mu l/10^8$ platelets	Platelet ^{22}Na 'space'
In the absence of ouabain (n = 36) [a]		0.518 ± 0.040 [c]	0.437 ± 0.031	0.081 ± 0.013
ADP (10^{-5} M)	60 s	0.512 ± 0.038	0.348 ± 0.026	0.163 ± 0.019 [d]
	90 s	0.515 ± 0.045	0.339 ± 0.031	0.175 ± 0.019 [d]
	180 s	0.476 ± 0.037	0.322 ± 0.026	0.154 ± 0.018 [d]
In the presence of ouabain (10^{-6} M) (n = 14) [b]		0.573 ± 0.035	0.328 ± 0.023	0.244 ± 0.019
ADP (10^{-5} M)	60 s	0.620 ± 0.059	0.300 ± 0.041	0.320 ± 0.029 [e]
	120 s	0.609 ± 0.057	0.261 ± 0.028	0.348 ± 0.033 [e]
	180 s	0.597 ± 0.047	0.266 ± 0.020	0.332 ± 0.031 [e]

[a] Number of determinations.
[b] Added 30 min before ADP.
[c] Mean ± standard error.
[d] ($P < 0.005$), statistical significance of mean relative to pre-ADP.
[e] ($P < 0.05$), statistical significance of mean relative to pre-ADP.

plasma in the platelet pellet compared to that in pellets of unaggregated platelets (Table I), presumably because of closer packing of aggregated platelets. A decrease in trapped plasma volume among aggregated platelets has been observed earlier [11] and is not unique to the use of ^{125}I-albumin as a marker (i.e., we find a similar decrease in trapped plasma using ^{14}C-labelled inulin). On the other hand, $^{22}Na^+$ radioactivity in the pellet of ADP-aggregated platelets

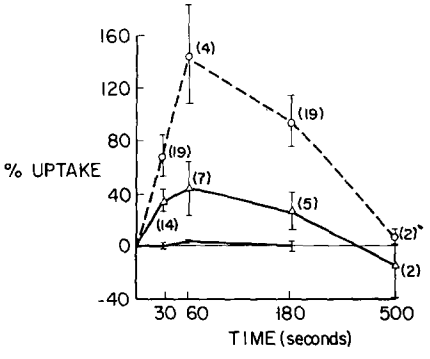

Fig. 3. Effect of different concentrations of ADP (shown as △ for $1 \cdot 10^{-6}$ and as ○ for $1 \cdot 10^{-5}$ M) and of GDP (● for $1 \cdot 10^{-5}$ M) on the ^{22}Na 'space' of platelets obtained from platelet-rich plasma incubated at 37°C. Other symbols as for Fig. 1. The ^{22}Na 'spaces' are expressed as per cent of the initial control value (0.095 ± 0.011 $\mu l/10^8$ platelet in 9 experiments).

TABLE II

EFFECT OF ADP ($10 \cdot 10^{-5}$ M) ON PLATELET ^{22}Na AND ^{36}Cl 'SPACES'

After addition of 10^{-5} M ADP to platelet-rich plasma, platelets were separated and the ^{22}Na and ^{36}Cl 'spaces' in the pellets were calculated as described in Table I.

	Control	10^{-5} M ADP		
		60 s	90 s $\mu l/10^8$ platelets	180 s
^{36}Cl ($n = 16$) [a]	0.355 ± 0.026 [b]	0.342 ± 0.024	0.340 ± 0.025	0.316 ± 0.032
^{22}Na ($n = 12$)	0.046 ± 0.011	0.102 ± 0.017 [c]	0.110 ± 0.017 [c]	0.102 ± 0.020 [c]

[a] Number of determinations.
[b] Mean ± standard error.
[c] ($P < 0.05$) statistical significance of mean relative to control.

remained approximately constant, indicating net uptake of $^{22}Na^+$. The direct determination of the Na^+ content of the platelet pellet by flame photometry confirmed that pellet Na^+ increased despite the fall in trapped volume (e.g., 14.7 ± 2.9% in 5 experiments). Table I shows that the platelet $^{22}Na^+$ 'space' was significantly increased 60, 90 and 180 s after the addition of ADP. This increase was transitory as measurements after 300 s (made in 4 experiments) showed that the $^{22}Na^+$ 'space' (0.088 ± 0.010 μl) decreased toward the control or pre-ADP value (0.100 ± 0.016 μl). In ouabain-treated platelets (10^{-6} M) the trapped plasma volume decreased in response to ADP while the $^{22}Na^+$ 'space' increased (Table I); however, the $^{22}Na^+$ 'space' remained elevated also after 300 s (0.385 ± 0.037 μl compared to the pre-ADP level of 0.205 ± 0.021 μl).

Although both $^{22}Na^+$ radioactivity and Na^+ concentration in platelets gradually increased with time of incubation, addition of ADP caused a further rise in $^{22}Na^+$, an increase in Na^+, and a decrease in K^+ concentration (Fig. 2A). These effects of ADP were also evident in ouabain-treated platelets despite the much larger accumulation of Na^+ that occurred during incubation with ouabain (Fig. 2B).

Fig. 3 shows that the extent of $^{22}Na^+$ uptake depended on the concentration of ADP because 10^{-5} M ADP induced a greater uptake than did 10^{-6} M. GDP (10^{-5} M), which does not induce aggregation, had no effect on $^{22}Na^+$ uptake.

The uptake of $^{22}Na^+$ brought about by ADP could represent the movement of Na^+ into the surface-connected canalicuar system or into the cytoplasm of the platelets. If the increase in $^{22}Na^+$ 'space' indicated $^{22}Na^+$ uptake into the canalicular system a corresponding increase in $^{36}Cl^-$ 'space' might be expected. However, aggregation by ADP did not cause such an increase in $^{36}Cl^-$ 'space' (Table II). As platelet aggregation is not associated with an increase in mean platelet volume [2,12], the influx of Na^+ without Cl^- is consistent with a selective mechanism for the movement of Na^+ into platelets rather than a movement of NaCl together with water under an osmotic gradient.

Discussion

The results show that, after an initial rapid exchange, unactivated human platelets exchange $^{22}Na^+$ slowly and that their activation by ADP is associated with a rapid influx of $^{22}Na^+$. This is not accompanied by a corresponding influx

of $^{36}Cl^-$, indicating that the effect of ADP is to accelerate an exchange affecting Na^+ rather than to initiate an osmotically determined movement of NaCl and water. This conclusion is in accord with the absence of an increase in mean platelet volume during platelet aggregation induced by ADP [2,12] and with the finding that ADP does not induce an uptake of $^{45}Ca^{2+}$ [17,18]. Our new observations suggest therefore, that activation of platelets, at least by ADP, is associated with a selective change in membrane premeability such that the movement of Na^+ into the cells down its electrochemical gradient is accelerated.

Platelets, like other cells, maintain steep gradients of Na^+ and K^+ concentrations across their membranes [6,16]. Presumably, also as in other cells, the movement of $^{22}Na^+$ into non-activated platelets represents inward diffusion of Na^+ which is pumped out by an Mg^{2+}-dependent ($Na^+ + K^+$)-activated ATPase in their membrane [9,10]. We found that in pletelet-rich plasma after an initial rapid exchange there was a slow movement of $^{22}Na^+$ into platelets in the first 2 h, with little change thereafter. The Na^+ entering the platelets represented 13.6% of their total Na^+ after 40 min and 31.6% after 120 min. In the same period the Na^+ concentration in the platelets increased by only about 21%. Thus, total Na^+ increased less rapidly than $^{22}Na^+$, indicating that there was an exchange of Na^+ between plasma and platelets.

Ouabain inhibits influx of K^+ into platelets [6,16]. Addition of ouabain to platelet-rich plasma caused rapid increases of both $^{22}Na^+$ and total Na^+ in the platelets, as well as a decrease in their content of K^+. Thus, in the presence of ouabain platelets gained Na^+ in exchange for K^+. Ouabain increased the fraction of total Na^+ in the platelets exchanged with $^{22}Na^+$ in 120 min to 57%.

Addition of ADP to platelet-rich plasma containing $^{22}Na^+$ caused a rapid rise in radioactivity of the platelets, an increase in their Na^+ and a decrease in their K^+; these changes coincided with aggregation. When ouabain was added first so that the platelets accumulated Na^+, and ADP was added later, there was a similar influx of $^{22}Na^+$. These effects can be explained in at least three different ways. First, ADP inhibits the Mg^{2+}-dependent ($Na^+ + K^+$) stimulated ATPase in the membrane so that Na^+ which diffused into the cells would not be pumped out. Thus, ADP inhibits the Mg^{2+}-dependent ($Na^+ + K^+$) stimulated *p*-nitrophenyl phosphatase activity [10] which is believed to represent the externally oriented ouabain-sensitive component of that ATPase system [19]. The proposition that this system is inhibited by ADP acting on the external surface of platelet membranes is consistent with evidence for the binding of ADP to specific receptors there [5].

Secondly, ADP might induce an increase in intracellular ionic calcium [20] which could inhibit the Mg^{2+}-dependent ($Na^+ + K^+$) stimulated ATPase [21] and/or induce an exchange of internal Ca^{2+} for external Na [22]. The Mg^{2+}-dependent ($Na^+ + K^+$) stimulated ATPase of erythrocytes is inhibited by Ca^{2+} [21]. On this analogy, if ADP were to induce an increase of ionized Ca in platelets, the efflux of Na^+ might be inhibited. It has been shown that activated muscle cells exchange internal Ca^{2+} with external Na^+. Thus, the rapid increase in $^{22}Na^+$ radioactivity caused by ADP would be due to inhibition of the Na^+ pump or to exchange of Ca_i^{2+} for Na_o^+.

Thirdly, ADP might increase the permeability of the platelet membrane to

Na^+. This possibility is supported by our observation that in the presence of ouabain ADP further increased $^{22}Na^+$ radioactivity and total Na^+ of platelets. It seems unlikely that ADP could further inhibit efflux of Na^+. Furthermore, if ADP inhibited efflux and the slow rate of inactivated Na^+ influx persisted, a rapid accumulation of $^{22}Na^+$ could not occur. Therefore, we favour the conclusion that ADP induces an influx of Na^+ rather than an additional inhibition of its efflux. Activation of Na^+ influx would enhance the resemblance of platelets to other excitable cells such as muscle, in which it is the primary excitatory process. On the other hand, inhibition of Na^+ efflux, by causing a rise in internal Na^+ could also be involved in the activation process in platelets.

Both ouabain and ADP increase Na^+ in platelets but only ADP causes them to aggregate [9]. Although this might conceivably be explained by differences in modes of action as discussed, a more probable conclusion is that the accumulation of Na^+ per se is not sufficient to induce platelet aggregation.

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