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# SEPARABLE FUNCTION OF PLATELET RELEASE REACTION AND CLOT RETRACTION

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SUMMARY: Amiloride, a known $Na^+/H^+$exchange inhibitor, inhibited platelet serotonin release in a dose-dependent manner (100μM for 50% inhibition, and 1mM for the nearly complete inhibition), although amiloride (1mM) accelerated clot retraction when it was measured at decreased platelet concentration. On the contrary, cytochalasin B (10μg/ml) accelerated platelet serotonin release, but it inhibited clot retraction. These results demonstrate that release reaction and clot retraction, both of which are important processes involved in platelet activation, can be functionally separated. 

Platelets respond to numerous stimuli with almost the same sequence of responses(1). The processes involved in platelet activation include shape change, aggregation, release reaction, and clot retraction. These events have been suggested to result from the activation and interaction of cytoskeletal proteins(2).

Carroll et al.(3) showed that there were separable assembly processes in platelets for pseudopodal and contractile gel formation by using the actin-polymerization inhibitor, cytochalasin B

Abbreviations: CB, Cytochalasin B; TPA, 12-O-tetradecanoylphorbol 13-acetate; PRP, Platelet-rich plasma; PPP, Platelet-poor plasma.

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(CB) and the platelet activator, 12-O-tetradecanoylphorbol 13-acetate (TPA). These results suggested that platelet activation processes could be separated by setting up proper conditions.

In the present study, we present data which show platelet release reaction and clot retraction are functionally separable by using CB and $Na^+/H^+$exchange inhibitor, amiloride.

## MATERIALS AND METHODS

Venous blood from healthy volunteers was collected into plastic tube containing 3.8%wt/vol sodium citrate (9 vol of blood for 1 vol of sodium citrate). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained as previously described (4). The release reaction was measured with platelets preloaded with [$^{14}$C] serotonin according to the method of Haslam et al.(5) Platelets were activated by ADP (10μM) and/or TPA (100ng/ml). PRP was preincubated with aspirin (100μg/ml) for 5 minutes at 37°C, and the release reaction was measured under the condition without stirring. Imipramine (1μM) was added just before the activation to prevent the reuptake of released serotonin. At 5 minutes after the stimulation, the reaction was terminated by 0.1%wt/vol glutaraldehyde (5 vol of PRP for 2 vol of glutaraldehyde). Clot retraction was measured with diluted PRP activated by thrombin (2.5U/ml) as described by Widmer et al.(6). Both CB and amiloride were dissolved in dimethylsulfoxide and diluted by saline when used. Pharmacological effect of these agents was evaluated by the preincubation with PRP for 15 minutes at 37°C.

[$^{14}$C]Serotonin was purchased from New England Nuclear. TPA was purchased from Pharmasia. Amiloride and CB were purchased from Sigma.

## RESULTS AND DISCUSSION

We evaluated serotonin release of aspirin-treated PRP under the condition without stirring to eliminate the secondary effect of aggregation or the formation of thromboxane $A_2$. Aspirin-treated PRP released little serotonin when it was stimulated by ADP (10μM) or TPA (100ng/ml) alone. But when ADP and TPA were added together, marked, more than additive release was observed (Fig. 1). This may reflect the synergistic effect of the ADP-induced activation of myosin light chain kinase which is responsible for the phosphorylation of myosin light chain (7) and TPA-induced activation of protein kinase C which is responsible for the phosphorylation of 40 kilodalton protein(8,9).

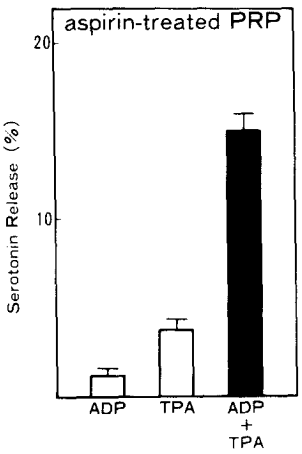


Figure 1. Synergistic effect of ADP (10μM) and TPA (100ng/ml) on serotonin release. PRP was incubated with aspirin (100μg/ml) for 5 minutes at 37°C prior to stimulation (ADP, TPA, or ADP+TPA). The reaction was induced without stirring. Percentages to the total counts were as follows (means±S.D.). ADP: 1.2±0.4% (n=5), TPA: 3.8±0.6% (n=5), ADP+TPA: 15.1±1.0% (n=6).

Recent studies suggest that amiloride, which is known as a potent inhibitor of $Na^+/H^+$exchange, may affect the functions of a variety of cells by inhibiting protein phosphorylation(10,11). Amiloride inhibited serotonin release measured by the above-mentioned method in a dose-dependent manner (100μM for 50% inhibition and 1mM for the nearly complete inhibition) (Fig. 2A). These results are compatible with previous studies reporting an inhibitory effect of amiloride on platelet secretion(12,13). This inhibitory effect of amiloride might be due to the inhibition of $Na^+/H^+$exchange across the membrane(14,15) or due to the inhibition of protein kinases such as protein kinase C(11) or myosin light chain kinase (Higashihara, M. unpublished data). On the contrary, CB potentiated the release reaction (Fig. 2B). Serotonin release with CB (10 μg/ml) was 2.8 times as much as that without CB. Haslam et al. (5) has already reported the similar result, but the precise mechanism of it is still unknown.

The effect of CB and amiloride on clot retraction was examined by the method described by Widmer et al. (Fig. 3A). CB (10

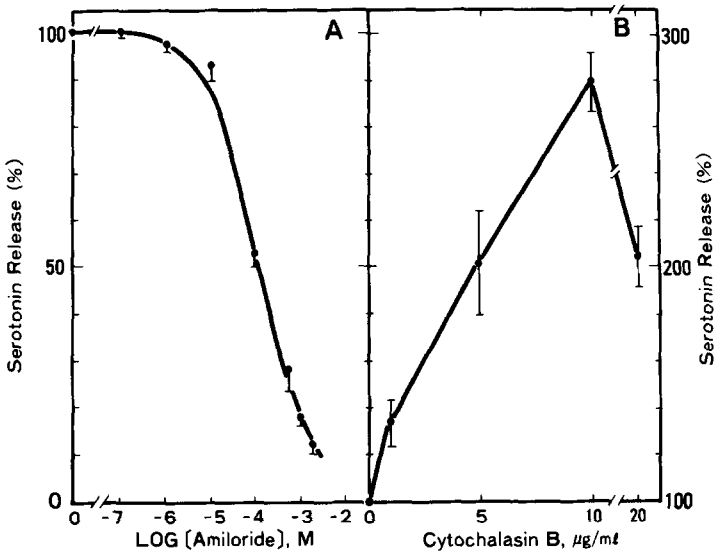


Figure 2. Inhibitory effect of amiloride (2A) and potentiating effect of CB (2B) on serotonin release induced by ADP+TPA. Amiloride or CB was preincubated for 15 minutes at 37°C. The amount of released serotonin was expressed as a percentage to that without each agent. Values are means±S.D. of four experiments.

μg/ml) exhibited inhibitory effect on clot retraction. Amiloride (1mM) did not exhibit striking effect, but small but significant ($p<0.02$) potentiating effect was observed for the first 15-30 minutes. To investigate it further, the platelet count was altered. When the platelet count was decreased and the progression of clot formation was retarded, the potentiating effect of amiloride (1mM) became more evident (Fig. 3B). Scanning electron microscopic examination revealed that CB markedly inhibited pseudopodal formation, but amiloride did not show this effect (data not shown). The inhibitory effect of CB on clot retraction might be due to the lack of pseudopodal development(16). The mechanism of potentiating effect of amiloride on clot retraction remains to be clarified.

In summary, CB potentiated platelet release reaction but inhibited clot retraction. On the contrary, amiloride inhibited the former but potentiated the latter. These results demonstrate that release reaction and clot retraction, both of which are im-

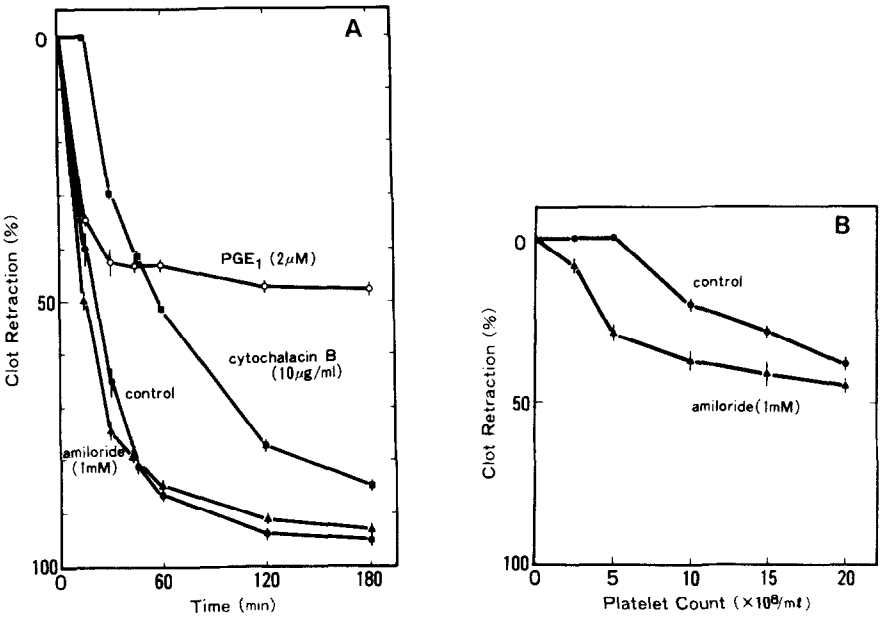


Figure 3A. The effect of $PGE_1$ (2μM), CB (10μg/ml), and amiloride (1mM) on clot retraction. Clot retraction was measured by the method as described by Widmer et al.(6). Amiloride or CB was preincubated for 15 minutes. $PGE_1$ was incubated for 5 minutes. Values are means±S.D. of three experiments. Inhibitory effect of CB (10μg/ml) on clot retraction is evident. Amiloride (1mM) potentiated clot retraction for the first 30 minutes. ($p<0.02$ both at 15 minutes and at 30 minutes)

Figure 3B. Comparison of clot retraction at various platelet counts. Retraction was determined and expressed as a percentage at 15 minute incubation. Accelerating effect of amiloride (1mM) on clot retraction is evident when the platelet count was decreased by adding PPP. Values are means±S.D. of three experiments. ($p<0.01$ at platelet count of 5, 10, and 20 x $10^8$/ml; $p<0.02$ at platelet count of 15 x $10^8$/ml)

portant processes involved in platelet activation, can be functionally separated, thus indicating these two processes have different activation mechanisms. It may be possible to speculate that actin-binding protein and myosin may compete for interaction with actin, considering that actomyosin contractile gel formation was responsible for release reaction(17) and the interaction between actin-binding protein and actin was responsible for pseudopodal formation(3,16).

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