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PARADOXICAL EFFECTS OF AMILORIDE ANALOGS ON PROTEIN PHOSPHORYLATION AND SEROTONIN RELEASE INDUCED BY AGONISTS IN HUMAN PLATELETS

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We examined the effects of newly exploited amiloride analogs on protein phosphorylation and serotonin secretion induced by various agonists in human platelets. 3', 4'-dichlorobenzamil (DCB) and to a lesser extent, 2', 4'-dimethylbenzamil (DMB), which in many cells highly specific inhibitors of Na^+/Ca^{2+}-pump, inhibited the phosphorylation of 47K- and 20K-dalton proteins and serotonin secretion in human platelets independently of the action on the pump. DCB also induced dephosphorylation of 47K and 20K after the phosphorylation of these proteins by thrombin and released serotonin by itself.

Thrombin and the calcium ionophore A23187 stimulate Na^+/H^+-pump on platelets (1-4), which is thought to play a role in some aspects of platelet activation including aggregation, microfilament formation, and activation of phospholipase A_2 (4-6). Amiloride analogs inhibit these processes (1-6). The introduction of a guanidine group in amiloride increases the specificity of this compound for the Na^+/Ca^{2+}-pump (7), whose physiological significance in platelets is yet

Abbreviations used are: DCB, 3', 4'-dichlorobenzamil; DMB, 2', 4'-dimethylbenzamil; IBA, 5-(N-methyl-isobutyl) amiloride; IPA, 5-(N-methyl-isopropyl) amiloride; PMA, 12, 13-phorbol myristate acetate.

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to be clarified. Recently, we reported that 5-(N-methyl-isobutyl) amiloride (IBA), and to a lesser digree, 5-(N-methyl-isopropyl) amiloride (IPA), highly specific inhibitors of the Na^+/H^+-pump, elicit phosphorylation and secretion independent of the action on the pump (8). In this communication, we present evidence that amiloride derivatives supposed to be highly specific for the Na^+/Ca^{2+} -pump (DCB, DMB) inhibit the phosphorylation and/or serotonin release induced by thrombin or other agonists independently of their possible action on the pumps. DCB also caused dephosphorylation.

Materials and Methods

Preparation of amiloride analogs---Amiloride analogs were synthesized as described elesewhere (9).

Preparation of washed platelets---Platelets were washed as described (10) and suspended at 2-4 x 10^8/ml in Tyrode Hepes buffer (140 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 5 mM N-2-Hydroxyethylpiperazine-N'-2 ethanesulfonic acid (Hepes), 1 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 0.1 mM $CaCl_2$, 1 mM glucose and 0.1 mg/ml apyrase (Sigma A6132).

SDS-polyacrylamide gel electrophoresis and phosphorylation ---Platelets were labelled with $[^{32}]P$ as previously described (10) and incubated with the analogs at 37°C, subjected to SDS-polyacrylamide gel electrophoresis according to Laemmli (11) using 13% slab gels, from which autoradiographs were prepared as described previously (10).

Serotonin release---Platelets were labelled with $[^{14}C]$-serotonin and the release induced by the analogs was determined as previously described (8).

Results and Discussions

Fig. 1 shows that preincubation of platelets with 0.1 mM DCB inhibited 82% of the secretion in response to thrombin (0.5 U/ml) followed by 55% inhibition by DMB. Other analogs at 0.1 mM did not inhibit except 18% inhibition by IBA. At 0.5 mM, DCB and DMB inhibited about 80% of the secretion, followed by 75% and 50% inhibition by IBA and IPA, respectively. The inhibition of secretion paralleled the suppression of the phosphorylation of 47K-and 20K-dalton proteins induced by thrombin as demonstrated in Fig. 2. DCB and ,to a lesser extent, DMB inhibited the phosphorylation (Lanes 2, 7).

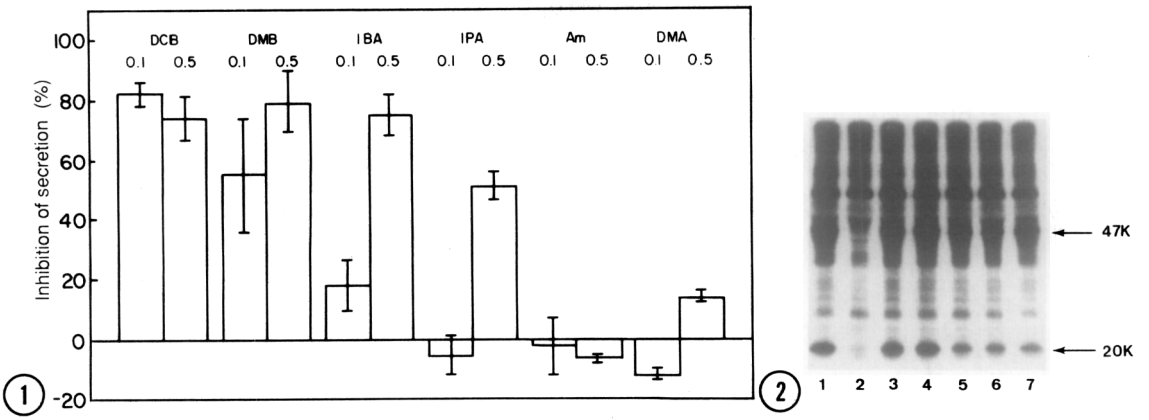
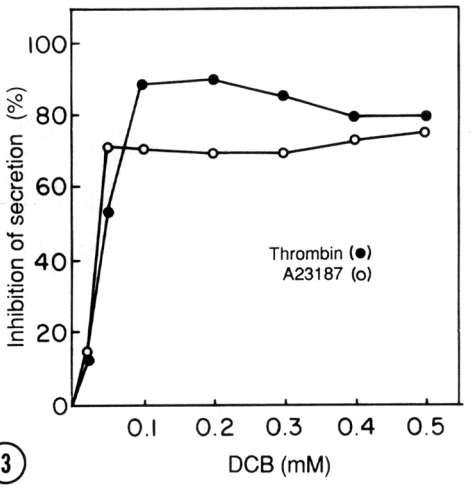


Fig. 1 Inhibition of thrombin-induced serotonin release by amiloride analogs
[^{14}C]-serotonin loaded platelets were incubated with either 0.1 mM or 0.5 mM amiloride analogs for 30 sec, then treated with 0.5 U/ml thrombin for 30 sec. Means ± S. D. of two experiments are shown (n=4).

Fig. 2 Inhibition of thrombin-induced phosphorylation by amiloride analogs
[^{32}P]-labelled platelets were treated with 0.2 mM amiloride analogs for 30 sec, then the phosphorylation was induced by 0.5 U/ml thrombin for 30 sec. Lane 1, control; Lane 2, DCB; Lane 3, amiloride; Lane 4, dimethylamiloride; Lane 5, IBA; Lane 6, IPA; Lane 7, DMB.

As shown in Fig. 3, serotonin release in response to thrombin and that to a Ca^{2+}-ionophore, A23187 was inhibited by DCB with the maximum inhibition at 0.1 mM and at 0.045 mM, respectively. The inhibition of serotonin release induced by thrombin was comparable with the inhibition of phosphorylation as depicted in Fig. 4. This is in line with Nishizuka's idea that the phosphorylation is a prerequisite for secretion, which is based on the temporal relationship between phosphorylation and secretion during platelet activation (12).

The inhibitory effect of DCB on phosphorylation was not mediated by the action of DCB on receptors since DCB inhibited the phosphorylation induced by the Ca^{2+}-ionophore (A23187), 12, 13-phorbol myristate acetate (PMA), and collagen as well as that by thrombin as shown in Fig. 5. On the other hand, DCB did not inhibit the secretion induced by PMA (Fig. 6) , which is slower and smaller (13) providing an exception to the Nishizuka's theory (12). As shown



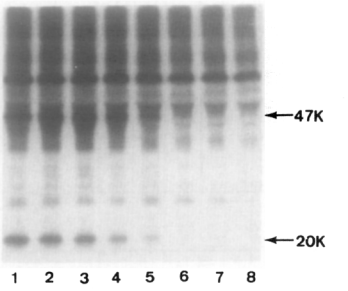
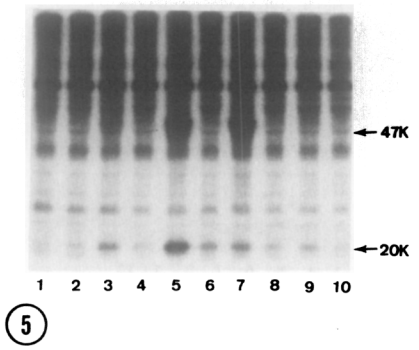


Fig. 3 Dose-dependent inhibition by DCB of serotonin secretion induced by thrombin or A23187
[^{14}C]-serotonin-loaded platelets were treated with various concentrations of DCB for 30 sec and then with 0.5 U/ml thrombin or 1 uM A23187 for 30 sec to induce secretion. Means of two experiments are shown.

Fig. 4 Dose-dependent inhibition by DCB on thrombin-induced phosphorylation
[^{32}P]-labelled platelets were treated with various concentrations of DCB for 30 sec and then with 0.5 U/ml thrombin for 30 sec. Concentrations of DCB are 0 (Lane 1), 10 uM (Lane 2), 20 uM (Lane 3), 50 uM (Lane 4), 100 uM (Lane 5), 200 uM (Lane 6), 300 uM (Lane 7), 500 uM (Lane 8).

in Fig. 6, the inhibition by DCB on thrombin-induced secretion was paradoxically decreased with higher concentrations of DCB or for longer incubation. This was found to be because DCB by itself evoked slow serotonin release (35% and 70% secretion by 0.1 mM and 0.5 mM DCB, respectively for 5 min) (Fig. 6) without releasing lactate dehydrogenase (data not shown). Indomethacin, apyrase, EGTA, or deoxyglucose did not inhibit the DCB-induced release. However, chilling the platelets almost completely inhibited the secretion (not shown). The DCB-induced secretion as well as the inhibition by DCB of thrombin-induced secretion are not likely to be mediated by the inhibition of the Na^+/Ca^{2+}-pump because, as shown in Fig. 7, treatment of platelets with saponin at a concentration (20 ug/ml) which allows selective permeabilization of plasma membrane (10) did not change the effects of DCB.



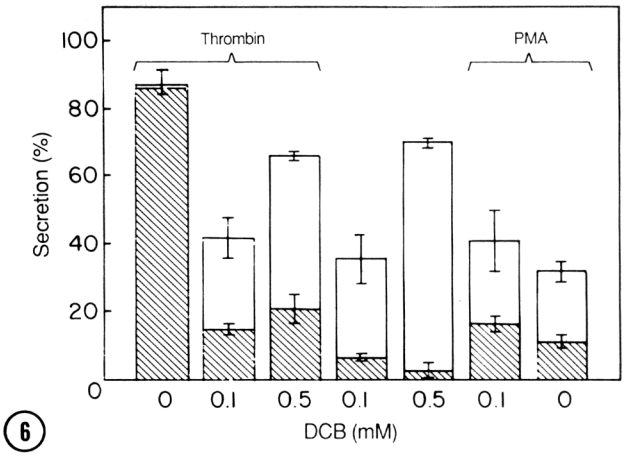


Fig. 5 Effect of DCB on the phosphorylation induced by various agonists
[32P]-labelled platelets were treated with various agonists after pre-exposure to 0.2 mM DCB for 30 sec. Lanes 1, 3, 5, 7, 9 are without pre-exposure to DCB and Lanes 2, 4, 6, 8, 10 are after preincubation with 0.2 mM DCB. Lanes, 1, 2, control; Lanes 3, 4, 20 uM ADP; Lanes 5, 6, 1 uM A23187; Lanes 7, 8, 50 nM PMA; Lanes 9, 10, 10 ug/ml collagen.

Fig. 6 Effect of DCB on serotonin release induced by thrombin or PMA.
[14C]-serotonin loaded platelets were treated with either 0.1 mM or with 0.5 mM DCB for 30 sec, then incubated with either 0.5 U/ml thrombin or 50 nM PMA. The whole columns represent the secretion after 5 min, of which hatched columns show the secretion after one min. Means ± S. D. of two experiments were shown (n=6).

How might DCB inhibit the agonist-induced secretion and phosphorylation? Inclusion of ATP to saponized-platelets did not relieave the inhibitory effect of DCB on thrombin-stimulated secretion (Fig. 7) although competitive inhibition by amiloride on ATP binding to kinases has been suggested (14). The possibility that DCB inhibited protein kinases (14, 15) still cannot be neglected, however, DCB did not inhibit protein kinase C, which phosphorylates 47K (10, 12), in platelet lysate (not shown).

As shown in Fig. 8, when DCB was added after thrombin had phosphorylated 47K- and 20K-dalton proteins, DCB dephosphorylated these proteins suggesting the presence of an amiloride-sensitive phosphatase as reported for hepatocytes (16) (compare lanes 5, 10 with lanes 6-9). The presence of the phosphatase was also suggested

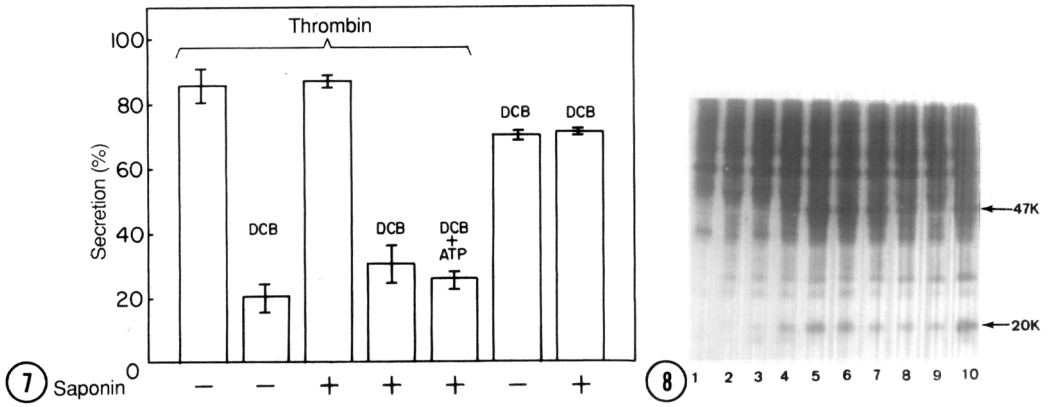


Fig. 7 Effect of saponization on the effect of DCB
Serotonin release induced by 0.5 U/ml thrombin for one min was determined after treating platelets with saponin (20 ug/ml) or water for two min. 0.1 mM DCB and/or 5 mM ATP was added 30 sec before adding thrombin. The serotonin secretion induced by 0.5 mM DCB for 5 min was also measured with or without saponin. Means ± S.D. (n=3) are shown.

Fig. 8 Effect of DCB added at various times on thrombin-induced phosphorylation
0.2 mM DCB was added at various timing and the phosphorylation induced by 0.5 U/ml thrombin was examined. Lane 1, no DCB, no thrombin; Lanes 2, 3, DCB for 5 min (2) or for 30 sec (3), then thrombin for one min; Lane 4, DCB and thrombin simultaneously for one min; Lane 5, thrombin for one min; Lane 6-9, thrombin for one min followed by DCB for 30 sec (6), 1 min (7), 2 min (8), or for 4 min (9); Lane 10, thrombin for 5 min.

in platelets treated with high concentrations of saponin in the presence of mM calcium (17).

Secretion can be induced independently of phosphorylation under some conditions found by ourselves and by others: 1) secretion induced by PMA is only slightly affected by DCB while phosphorylation was inhibited (Fig. 5, 6); 2) DCB by itself caused secretion without phosphorylation (Fig. 5, 6); 3) IBA elicits secretion without phosphorylation in the presence of an ADP scavenger (8); 4) thrombin caused secretion without the phosphorylation in saponized platelets in the presence of mM calcium as suggested by Lapetina et al (17); and 5) calcium ionophore caused secretion and calcium release without the phosphorylation in the presence of indomethacin and ADP scavengers as reported by Rittenhouse and Horne (18). On the other hand, at least some portion of the 47K-

dalton protein is shown to be inositol trisphosphate 5'-phosphomonoesterase (19) and the phosphorylation of 20K-dalton protein (myosin light chain) by protein kinase C was reported to be inhibitory for secretion (13). Thus, a conclusive role for the phosphorylation of 47K and 20K in secretion is yet to be elucidated. On the other hand, our results clearly showed that DCB and DMB have at least two effects other than their reported effects on the Na^+/Ca^{2+} pump.

Acknowledgement

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