Effects of proteolysis on the actions of monovalent cations and 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine on platelet adenylate cyclase

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NaCl stimulated the adenylate cyclase activities of human and rabbit platelet particulate fractions prepared in the presence of ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate, but inhibited the activities of particulate fractions proteolysed by endogenous Ca^{2+}-activated protease or treatment with α-chymotrypsin. Studies with other monovalent cations showed that LiCl had weak effects similar to those of NaCl, whereas KCl inhibited the enzyme in both proteolysed and non-proteolysed preparations. The results suggest that NaCl exerts stimulatory and inhibitory effects through different sites. NaCl potentiated and proteolysis greatly reduced the inhibition of platelet adenylate cyclase by 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine (platelet-activating factor).

Adenylate cyclase *Proteolysis* *NaCl* *1-O-octadecyl-2-O-acetyl-sn-glyceryl-3-phosphorylcholine*

1. INTRODUCTION

Treatment of a variety of cells and membrane preparations with proteolytic enzymes has been reported to enhance basal adenylate cyclase activity and to modify the regulation of this enzyme by hormones and guanine nucleotides [1]. In the case of the rat platelet, incubation of homogenate with $CaCl_2$ caused a 2–7-fold increase in enzyme activity that could be blocked by leupeptin or *N*-ethylmaleimide, indicating that this effect was attributable to the action of an endogenous Ca^{2+}-dependent thiol protease [2]. In addition, experiments with human platelets have demonstrated that exogenous serine proteases block the inhibition of PGE_1-stimulated [3] and basal [4] adenylate cyclase activities by epinephrine. These effects were attributed to loss of the function of the N_i protein that couples inhibitory receptors to the catalytic moiety of adenylate cyclase [3,4]. In contrast, receptor-mediated activation of the enzyme was unimpaired by proteolysis [3].

We have shown that 1-octadecyl-2-acetyl-G-3-PC inhibits adenylate cyclase activity in particulate fractions from both human and rabbit platelets [5]. This inhibition was observed with nanomolar concentrations of the compound and was structurally highly specific, suggesting that it was a receptor-mediated effect similar to that of epinephrine. However, the maximal inhibitions of basal activity observed were variable and relatively low (20–50%). Since limited proteolysis can suppress the effects of inhibitory hormones on adenylate cyclase, we have investigated the possibility that endogenous proteolysis restricted the inhibition caused by this compound. In many biological systems, NaCl potentiates hormonal inhibition of adenylate cyclase by stimulating basal enzyme activity in the presence of GTP, an effect that is reversed by the inhibitory hormone [6,7]. However, studies with the platelet system have in-

Abbreviations: 1-octadecyl-2-acetyl-G-3-PC, 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid

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dicated that NaCl does not potentiate the inhibitory action of epinephrine and inhibits basal and PGE_1-stimulated adenylate cyclase activity [8,9]. In an attempt to enhance the inhibitory effect of 1-octadecyl-2-acetyl-G-3-PC, we have reinvestigated the action of NaCl on platelet adenylate cyclase and have found that the effects observed depend critically on whether or not the platelet membranes have been exposed to proteolytic enzymes.

2. MATERIALS AND METHODS

2.1. *Materials*

Synthetic 1-octadecyl-2-acetyl-G-3-PC was a generous gift from Dr H.R. Baumgartner of F. Hoffman-La Roche (Basel). Tris–ATP, Tris–phosphocreatine, α-chymotrypsin, soybean trypsin inhibitor and leupeptin were obtained from Sigma (St. Louis, MO). [2,8-^{3}H]ATP (26.6 Ci/mmol) was obtained from ICN (Irvine, CA). All other materials were obtained from sources listed in [5].

2.2. *Preparation of platelet particulate fractions and assay of adenylate cyclase*

Rabbit and human platelets were isolated and washed as in [5] and were finally resuspended at 25 mg wet wt/ml in 150 mM Tris–HCl (pH 7.4) containing either no other additions, 5 mM EGTA or 400 μM leupeptin. After freezing and thawing these suspensions and treatment of the lysates as described in the figure and table legends, the particulate fractions were isolated by centrifugation at 37500 × g for 40 min at 4°C and resuspended for assay of adenylate cyclase as in [5]. In addition to the compounds under investigation, adenylate cyclase assay mixtures (250 μl) all contained 75 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 0.4 mM Tris–[^{3}H]ATP, 4 mM Tris–phosphocreatine, 20 units of creatine phosphokinase/ml, 1 mM cAMP, 1 mM 3-isobutyl-1-methylxanthine, 1 mg of crystallized bovine serum albumin/ml, 0.4 mM dithiothreitol, 10 μM GTP and 0.4 mM Tris–EGTA. After incubation for 10 min at 30°C, assays were terminated and [^{3}H]cAMP isolated and quantified [5]. The platelet protein present was measured as in [5]. All adenylate cyclase assays were carried out in triplicate; activities are given as means ± SE.

3. RESULTS

The basal adenylate cyclase activity of particulate fractions from human platelets lysed in the absence of EGTA was about 3 times higher than the activity of fractions prepared in the presence of EGTA (table 1). NaCl had opposite effects on the activities of these two preparations, inhibiting the enzyme when EGTA was omitted and stimulating when EGTA was used. Addition of EGTA to the platelet lysis buffer and inclusion of NaCl in the assay medium both resulted in an enhanced inhibition of human platelet adenylate cyclase by 1-octadecyl-2-acetyl-G-3-PC (table 1). Thus, 100 mM NaCl increased the inhibition caused by 400 nM 1-octadecyl-2-acetyl-G-3-PC from 16 to 35% when EGTA was omitted, and from 28 to 68% when EGTA was used. PGE_1 increased the adenylate cyclase activities of particulate fractions prepared with or without EGTA to similar extents. However, the stimulatory effect of NaCl was largely lost in the presence of PGE_1, although its inhibitory action was undiminished. The percentage inhibition of adenylate cyclase by 1-octadecyl-2-acetyl-G-3-PC was always reduced in the presence of PGE_1 (table 1). The effects of use of EGTA on both basal adenylate cyclase activity and the inhibitory action of 1-octadecyl-2-acetyl-G-3-PC suggested that significant Ca^{2+}-dependent proteolysis occurred when platelets were lysed in the absence of this compound.

More detailed studies were carried out with the rabbit platelet enzyme. First, the actions of EGTA were compared with those of leupeptin to confirm that the former compound acted by inhibiting proteolysis. In the experiment shown in fig.1, platelet lysates were incubated for 15 min at 25°C to enhance any endogenous proteolysis. Under these conditions, particulate fraction from the control lysate had a basal adenylate cyclase activity 5-times higher than particulate fractions from lysates containing either 5 mM EGTA or 400 μM leupeptin. Addition of 10–150 mM NaCl inhibited the activity of the proteolysed preparation (maximum 45% at 50 mM) (fig.1A), but stimulated that of preparations from platelets lysed in the presence of either EGTA or leupeptin by up to ten-fold (fig.1B,C). With proteolysed preparations, NaCl often had a biphasic effect, causing substantially less inhibition in the presence of 150 mM NaCl

Table 1

Effects of NaCl and 1-octadecyl-2-acetyl-G-3-PC on the adenylate cyclase activities of human platelet particulate fractions prepared in the presence and absence of EGTA

Enzyme preparation	Additions	Adenylate cyclase activity (pmol · min^{-1} · mg protein^{-1})		Inhibition of adenylate cyclase by 1-octadecyl-2-acetyl-G-3-PC (%)
		Without 1-octadecyl-2-acetyl-G-3-PC	With 1-octadecyl-2-acetyl-G-3-PC	
+ EGTA	None	11.9 ± 0.2	8.6 ± 0.2	28 ± 2
	NaCl	35.3 ± 1.1	11.2 ± 0.1	68 ± 1
	PGE_1	138.9 ± 5.4	106.7 ± 0.5	23 ± 3
	NaCl + PGE_1	150.7 ± 1.7	82.6 ± 1.3	45 ± 1
− EGTA	None	31.7 ± 1.3	26.5 ± 0.6	16 ± 4
	NaCl	19.1 ± 0.7	12.5 ± 0.6	35 ± 4
	PGE_1	373.1 ± 12.2	359.1 ± 18.7	4 ± 6
	NaCl + PGE_1	170.2 ± 5.5	154.3 ± 1.0	9 ± 3

Human platelets were lysed by freezing and thawing in the presence or absence of 5 mM EGTA and the particulate fractions were isolated immediately. Adenylate cyclase assays were carried out with the additions indicated. Final concentrations were: NaCl, 100 mM; PGE_1, 200 nM; 1-octadecyl-2-acetyl-G-3-PC, 400 nM. Values are means ± SE from triplicate assays

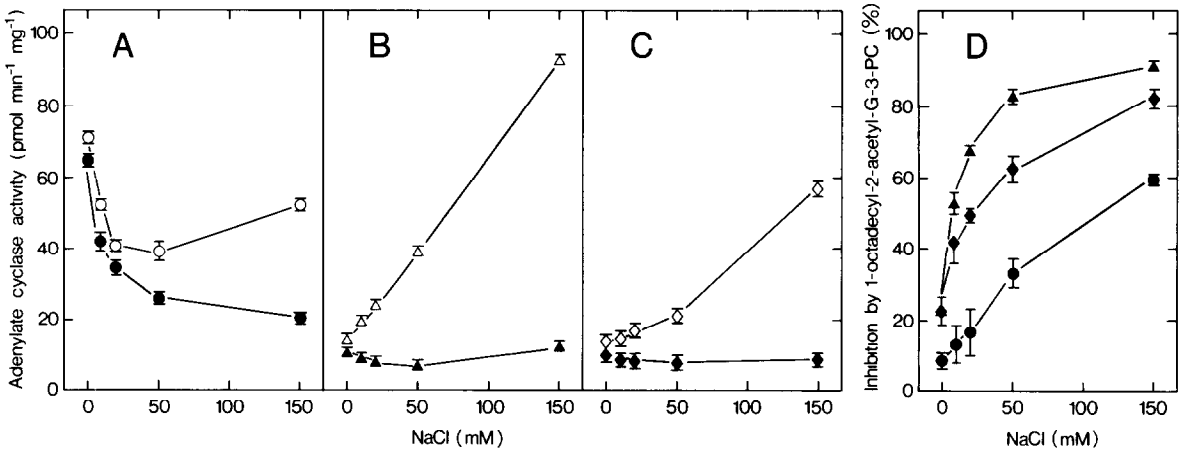

Fig.1. Effects of NaCl and 1-octadecyl-2-acetyl-G-3-PC on the adenylate cyclase activities of particulate fractions prepared from rabbit platelets lysed in 150 mM Tris–HCl (pH 7.4), containing no other additions (A), 5 mM EGTA (B) or 400 μM leupeptin (C). All three lysates were incubated for 15 min at 25°C before isolation of the particulate fractions. Assays were carried out in the absence (open symbols) and presence (closed symbols) of 100 nM 1-octadecyl-2-acetyl-G-3-PC. Inhibition of adenylate cyclase activity by this compound is shown in D, with additions during platelet lysis as follows: (●) none (from A); (▲) EGTA (from B); (◆) leupeptin (from C).

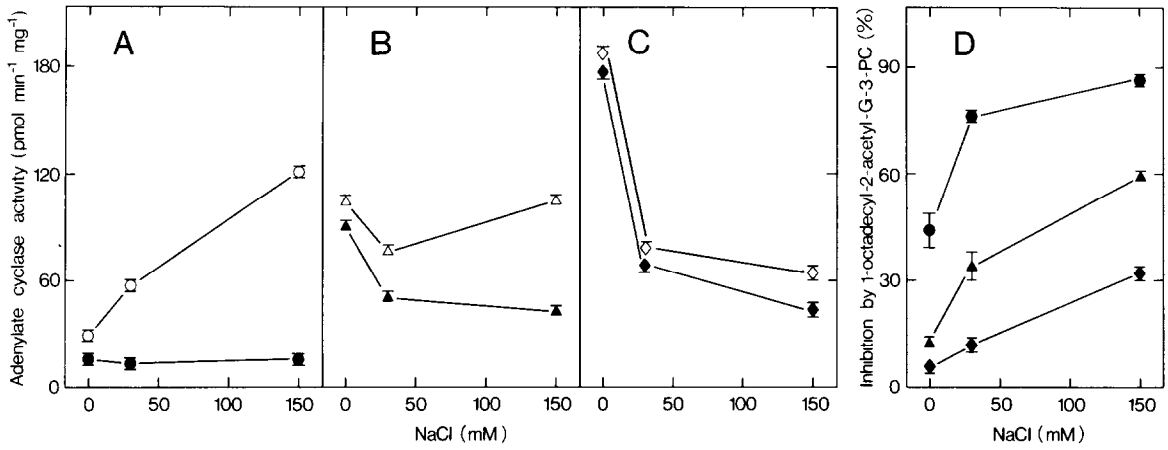

Fig.2. Effects of α-chymotrypsin on the actions of NaCl and 1-octadecyl-2-acetyl-G-3-PC on the adenylate cyclase activities of rabbit platelet particulate fractions. Platelet lysate containing 5 mM EGTA was incubated for 10 min at 25°C with no additions (A), 20 μg of α-chymotrypsin/ml (B) or 50 μg of α-chymotrypsin/ml (C). These incubations were terminated by addition of 500 μg of soybean trypsin inhibitor/ml and after 2 min the mixtures were cooled to 0°C. Particulate fractions were then isolated by centrifugation (38000 × g for 40 min) and washed twice by homogenization and re-centrifugation in buffer containing 10 mM Tris–HCl and 5 mM EGTA, pH 7.4. Assays were carried out in the absence (open symbols) and presence (closed symbols) of 100 nM 1-octadecyl-2-acetyl-G-3-PC. Inhibition of adenylate cyclase activity by this compound is shown in D for the following additions during incubations of the platelet lysate: (●) none (from A); (▲) 20 μg of α-chymotrypsin/ml (from B); (◆) 50 μg of α-chymotrypsin/ml (from C).

than 50 mM NaCl (fig.1A). Addition of 100 nM 1-octadecyl-2-acetyl-G-3-PC blocked the stimulation of adenylate cyclase by NaCl seen in particulate fractions prepared with EGTA or leupeptin, with the result that inhibition of the enzyme increased from 20–30% in the absence of NaCl to 80–90% in the presence of 150 mM NaCl (fig.1B,C). The salt concentration required for half-maximal inhibition of adenylate cyclase by 1-octadecyl-2-acetyl-G-3-PC was markedly increased by proteolysis (fig.1D).

Incubation of rabbit platelet lysate containing 5 mM EGTA with 50 μg of α-chymotrypsin/ml prior to isolation of the particulate fraction resulted in an up to 6-fold stimulation of basal adenylate cyclase activity (fig.2A,C). Under these conditions, NaCl caused a more pronounced inhibition of the enzyme than observed after endogenous proteolysis, probably because the stimulatory action of this salt was completely or almost completely lost. A biphasic effect of NaCl, similar to that usually observed after endogenous proteolysis, was obtained when a lower α-chymotrypsin concentration was used (fig.2B). Pre-incubation of lysate with α-chymotrypsin diminished the inhibition of adenylate cyclase activity by 1-octadecyl-2-acetyl-G-3-PC in a dose-dependent manner (fig.2D); at 50 μg of protease/ml, no inhibitory effect of this compound was detected in the absence of NaCl and only a weak inhibition was observed with 150 mM NaCl.

The specificity of these actions of NaCl on rabbit platelet adenylate cyclase was investigated by comparative studies with the chlorides of different monovalent cations (table 2). With non-proteolysed platelet particulate fractions, LiCl was stimulatory, although less so than NaCl, and KCl weakly inhibitory. However, with proteolysed preparations, both LiCl and KCl were inhibitory. Choline-Cl had no effect on either preparation. These results show that neither the stimulatory nor inhibitory action of NaCl was attributable to an increase in the ionic strength of the medium or to Cl^-. The effects of these salts on the adenylate cyclase activities of proteolysed and non-proteolysed human platelet particulate fractions (not shown) were very similar to those observed with rabbit material.

Table 2

Effects of different monovalent cations on the adenylate cyclase activities of non-proteolysed and proteolysed rabbit platelet particulate fractions

Expt.	Salt	Concentration (mM)	Adenylate cyclase activity (% change)	
			Non-proteolysed enzyme	Proteolysed enzyme
1	NaCl	30	+107 ± 5	−37 ± 1
		150	+489 ± 20	+18 ± 3
	LiCl	30	+ 22 ± 4	−17 ± 2
		150	+ 83 ± 5	−14 ± 3
	KCl	30	− 6 ± 3	−14 ± 1
		150	− 13 ± 2	−37 ± 5
	Choline-Cl	30	+ 1 ± 3	− 5 ± 2
		150	0 ± 3	− 8 ± 3
2	NaCl	30	+ 66 ± 7	−37 ± 4
		150	+295 ± 15	−24 ± 2
	LiCl	30	+ 6 ± 5	−15 ± 2
		150	+ 56 ± 5	−37 ± 2
	KCl	30	− 17 ± 3	−15 ± 2
		150	− 22 ± 3	−35 ± 2
	Choline-Cl	30	− 11 ± 5	− 5 ± 4
		150	− 9 ± 4	− 2 ± 4

In expt.1, platelets lysed in the presence or absence of EGTA were incubated for 15 min at 25°C before isolation of the particulate fractions. Control activities of non-proteolysed (+ EGTA) and proteolysed (− EGTA) particulate fractions were 19.6 ± 0.5 and 66.4 ± 0.3 pmol cAMP·min^{-1}·mg protein^{-1}, respectively. In expt.2, platelet lysate containing EGTA was incubated with or without 50 μg of α-chymotrypsin/ml for 10 min at 25°C before isolation of particulate fractions as described in the legend to fig.2. Control activities of non-proteolysed and proteolysed preparations were 40.3 ± 1.3 and 162 ± 3 pmol cAMP·min^{-1}·mg protein^{-1}. All values are means ± SE from triplicate assays

4. DISCUSSION

Omission of EGTA from the platelet lysis buffer had 3 major effects on the adenylate cyclase activities of human and rabbit platelet particulate fractions: basal activity was increased, stimulation of activity by NaCl was replaced by inhibition and the inhibitory effect of 1-octadecyl-2-acetyl-G-3-PC was diminished. Several lines of evidence indicated that these changes in enzyme activity resulted from Ca^{2+}-dependent proteolysis of the platelet membrane. Thus, two forms of Ca^{2+}-dependent protease have been identified in platelets [10] and leupeptin, a potent inhibitor of this enzyme [11], had the same effects as chelation of platelet Ca^{2+} by EGTA. Moreover, incubation of the platelet lysate with α-chymotrypsin in the presence of EGTA led to essentially the same results as omission of EGTA. The results in [2], where it was found that incubation of platelet

lysate with added $CaCl_2$ enhanced adenylate cyclase activity, are in accord with our conclusion. Our findings suggest that the inhibition of adenylate cyclase by NaCl previously observed with membranes from platelets [8,9] and neuroblastoma × glioma hybrid cells [12] was due to limited proteolysis of these membranes during their isolation. It may be possible to use this inhibitory effect of NaCl on adenylate cyclase as an indicator of proteolytic membrane alteration in a variety of biological systems.

The two opposite effects of NaCl on platelet adenylate cyclase activity that we have observed, together with their different cation specificities, suggest that NaCl may act at two different sites. This conclusion is consistent with evidence that NaCl inhibits the effects of both the N_i and N_s GTP-binding proteins in adipocyte membranes [13]. Several studies have shown that limited proteolysis can selectively eliminate the function of the N_i protein [3,4,13]. Our results are in agreement with these findings since the inhibitory action of 1-octadecyl-2-acetyl-G-3-PC on platelet adenylate cyclase, which we have shown to be GTP-dependent [14], was largely abolished by proteolysis, whereas the stimulatory action of PGE_1 was unaffected. Because inhibition by NaCl was only observed after proteolysis, when N_i-mediated effects were absent or reduced, it is possible that this action of NaCl is attributable to inhibition of N_s function. Conversely, the stimulatory effect of NaCl, which was reversed by 1-octadecyl-2-acetyl-G-3-PC, could be exerted through N_i, as suggested in [13]. However, this hypothesis does not explain why the stimulation of basal adenylate cyclase activity by NaCl requires a lower GTP concentration than the reversal of this effect by 1-octadecyl-2-acetyl-G-3-PC [14].

These studies show the importance of eliminating Ca^{2+}-dependent proteolysis during and after lysis of platelets in obtaining an optimal hormonal inhibition of particulate fraction adenylate cyclase. They also show, for the first time, that NaCl stimulates the activity of the platelet enzyme, provided proteolysis is prevented, and enhances the inhibitory effect of 1-octadecyl-2-acetyl-G-3-PC. Under optimal conditions, this agonist caused up to 68 and 90% inhibitions of the adenylate cyclase activities of human and rabbit preparations, respectively.

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