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INHIBITORY EFFECT OF 8-BROMO CYCLIC GMP ON AN EXTRACELLULAR Ca^{2+}-DEPENDENT ARACHIDONIC ACID LIBERATION IN COLLAGEN-STIMULATED RABBIT PLATELETS

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Abstract—The inhibitory effect of cyclic GMP on collagen-induced platelet activation was studied using 8-bromo cyclic GMP (8brcGMP) in washed rabbit platelets. Addition of collagen (1 μg/ml) to platelet suspension caused shape change and aggregation associated with thromboxane (TX) A_2 formation. 8brcGMP (10–1000 μM) inhibited collagen-induced platelet aggregation and TXA_2 formation in a concentration-dependent manner. 8brcGMP did not affect platelet cyclooxygenase pathways, but markedly inhibited collagen-induced arachidonic acid (AA) liberation from membrane phospholipids in [^{3}H]AA-prelabeled platelets, indicating that the inhibitory effect of 8brcGMP on collagen-induced aggregation is due to an inhibition of AA liberation. In [^{32}P]orthophosphate-labeled platelets, collagen stimulated phosphorylation of a 20,000 dalton (20-kD) and 40-kD proteins. 8BrcGMP stimulated phosphorylation of a specific protein having molecular weight of 46-kD and inhibited collagen-induced both 20- and 40-kD protein phosphorylation. Collagen could stimulate the AA liberation without activation of phospholipase C or Na^+–H^+ exchange, but could not in the absence of extracellular Ca^{2+}. These findings suggest that cyclic GMP inhibits collagen-induced AA liberation which is mediated by an extracellular Ca^{2+}-dependent phospholipase A_2. However, cyclic GMP seems to inhibit the Ca^{2+}-activated phospholipase A_2 indirectly, since 8brcGMP had no effect on Ca^{2+} ionophore A23187-induced platelet aggregation or AA liberation. It is therefore suggested that cyclic GMP may regulate collagen-induced increase in an availability of extracellular Ca^{2+} which is responsible for phospholipase A_2 activation in rabbit platelets.

In platelets, cyclic GMP is accumulated during platelet aggregation in response to several agonists [1–3]. It was originally thought that the increase in cyclic GMP levels might lead to platelet activation [1–4]. Recent studies from this and other laboratories, however, have suggested that cyclic GMP acts as an inhibitory mediator against platelet activation [5, 6]. This concept comes from the findings that stimulators of platelet guanylate cyclase, such as nitroprusside and 8brcGMP inhibit platelet aggregation and cause a disaggregation of aggregated platelets [5, 6]. More recently, both nitroprusside and 8brcGMP have been shown to attenuate protein kinase C activation [7] and elevation of cytoplasmic Ca^{2+} [8, 9] by inhibiting production of the second messenger molecules, diacylglycerol [7, 8] and inositol trisphosphate [9] in thrombin-activated human platelets, respectively. Therefore, one of the mechanisms of the inhibitory effect of cyclic GMP in platelets has been suggested to be due to an inhibition of phosphoinositide hydrolysis by the specific phospholipase C [7–9].

Although several platelet agonists such as thrombin, platelet-activating factor and TXA_2 mimetics have been reported to induce their stimulatory effect through the receptor-coupled phospholipase C activation [10–12], collagen at low concentration has been reported to induce platelet aggregation, release reaction and phosphoinositide hydrolysis by a mechanism which is completely dependent on the cyclooxygenase pathway [14], meaning that liberation of AA, the precursor for TXA_2, from membrane phospholipids is also an important step for the initiation of platelet function. Thus, determination of the effect of cyclic GMP on AA liberation in activated platelets would provide further information regarding the role of cyclic GMP in the regulation of platelet function. In the present study, we show that collagen-induced AA liberation, which is mediated by an extracellular Ca^{2+}-dependent phospholipase A_2, is inhibited by 8brcGMP.

MATERIALS AND METHODS

Washed rabbit platelets were prepared as previously described [6, 15]. Platelets were finally suspended at 7–9 × 10^8 cells/ml in a Hepes buffer consisting of 130 nM NaCl, 4.7 mM KCl, 4.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.0 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11.5 mM dextrose, 0.2% bovine serum albumin and 10 mM Hepes (pH 7.4). In some experiments, platelets were suspended in a Na-free Hepes buffer in which NaCl and $NaHCO_3$ were replaced with 140 mM *N*-methyl-D-glucamine and 4.0 mM $KHCO_3$, respectively. Platelet aggregation was measured turbidimetrically in an aggregometer (RAM-31; Rikadenki Kogyo Co., Ltd, Japan) as previously described [6, 15].

Measurement of TXB_2. Samples of platelet suspension (1.0 ml) were placed in aggregometer cuvettes and preincubated for 3 min at 37° in the aggregometer. Platelets were then stimulated with collagen and light transmission was continuously recorded. At the indicated time, an aliquot (0.1 ml) was

withdrawn from the cuvette and mixed with ice-cold solution containing 0.1 ml of 50 μM indomethacin and 50 mM EDTA. Following centrifugation at 1500 g for 10 min, the supernatant (0.1 ml) was diluted with 10 mM Tris–HCl (pH 7.6) and TXB_2 content was measured by radioimmunoassay. Samples (0.1 ml) or standard solutions (6.25–1600 pg/0.1 ml of 10 mM Tris–HCl, pH 7.6) were incubated with 0.1 ml of [^{3}H]TXB_2 (10 nCi) and 0.1 ml of TXA_2-antiserum at dilution (1:20,000) for 18–24 hr at 4°. The incubation was terminated by addition of an ice-cold solution of dextran-coated charcoal (0.5 ml) and centrifuging at 1500 g for 5 min at 4°. The antibody-bound [^{3}H]TXB_2 in the supernatant was measured by liquid scintillation counting. The antiserum to TXB_2 was kindly provided by Ono Pharmaceutical Co. Ltd (Japan) and its cross-reactivities for other AA metabolites were less than 0.1%.

[^{14}C]*Arachidonic acid release*. Platelets were labeled with [^{14}C]AA as previously described [15]. Samples (0.5 ml) of [^{14}C]AA-labeled platelets were preincubated for 3 min at 37° in the aggregometer and then stimulated with collagen or A-23187. The reaction was terminated by adding 0.5 ml of 50 μM indomethacin and 50 mM EDTA. After centrifugation at 1,700 g for 10 min, radioactivity of a 0.5 ml aliquot of the supernatant was measured by liquid scintillation counting.

Measurement of [^{3}H]*inositol phosphates*. Platelets suspended in a Ca^{2+}-free Hepes buffer (pH 7.4) were labeled with [^{3}H] inositol (30 μCi/ml) for 120 min at 37°, and then washed twice and resuspended in the buffer as described above. Samples (0.5 ml, 1–2 $\times$ 10^9 platelets) were placed in aggregometer cuvettes and preincubated for 10 min at 37° in the presence of 10 mM LiCl. Platelets were then stimulated with collagen. Platelet shape change and aggregation were recorded in the aggregometer. LiCl did not affect platelet response to collagen. The reaction was terminated by adding 0.5 ml of ice-cold trichloracetic acid (10% w/v). The platelets were then sonicated at 4° for 15 sec by a sonicator (UR-20P, Tomy Seiko Co. Ltd, Japan). After centrifugation at 1700 g for 10 min, the supernatants were washed four times with 2 ml of ethylether to remove trichloracetic acid. Each sample was then applied to a column containing 1 ml bed volume of anion exchange resin (Bio-Rad AG 1-X8, 100–200 mesh, formate form) [16]. The columns were washed with 10 ml of water and 10 ml of 50 mM ammonium formate to remove [^{3}H]inositol and glycerophosphoinositol, respectively. Then, inositol phosphates (IPs), containing inositol monophosphate, inositol bisphosphate and inositol trisphosphate, were eluted with 8 ml of 1 M ammonium formate/100 mM formic acid. Radioactivity of the elution was measured by liquid scintilation counting.

Protein phosphorylation. Platelets suspended in a Ca^{2+}-free Hepes buffer (pH 7.4) were labeled with [^{32}P]orthophosphate (0.2 mCi/ml) for 60 min at 37°, and then washed twice and resuspended in the buffer as described above. Samples (0.25 ml) were placed in aggregometer and stimulated with collagen. The reaction was terminated by addition of 0.25 ml of Laemmli sample buffer [17] and heated at 90° for 10 min. Aliquots containing 10–20 μg of protein were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide slab gel electrophoresis (PAGE) using the Laemmli buffer [17]. The stacking and separating gels contained 3.5 and 11.5% acrylamide, respectively. Gels were stained with Coomassie Brilliant Blue and dried. Phosphorylated protein bands on the gels were detected by autoradiography using Kodak X-Omat AR films. Molecular weights of the proteins were determined after SDS–PAGE using standards obtained from Pharmacia (LMW kit E).

Materials. Collagen was obtained as a 1 mg/ml suspension from Horman-Chemie Müchen GmbH. [1-^{14}C]AA (58 mCi/mmol) was obtained from Amersham Corp. [^{3}H]inositol (12.8 Ci/mmol) and carrier-free [^{32}P]orthophosphate were from New England Nuclear. Other reagents used were 8-bromo cyclic GMP (Sigma), dibutyryl cyclic AMP (Sigma), *N*-methyl-D-glucamine (Sigma), indomethacin (Sumitomo) and A-23187 (Calbiochem).

RESULTS

Effects of 8brcGMP *on collagen-induced aggregation and* TXB_2 *formation*

Stimulation of rabbit platelets with collagen (1 μg/ml) induced platelet shape change, aggregation and TXB_2 formation after a lag period of 30–45 sec (Fig. 1). The onset of shape change and development of aggregation were closely associated with TXB_2 formation. Indomethacin (2.5 μM) completely inhibited collagen-induced aggregation and TXB_2 formation, indicating that collagen at the concentration of 1 μg/ml caused platelet activation through a mechanism requiring TXA_2 formation from endogenous AA. 8brcGMP (1 mM) had a potent inhibitory effect on both collagen-induced aggregation and TXB_2 formation (Fig. 1). The effects of different concentrations of 8brcGMP on collagen-induced aggregation and TXB_2 formation was shown in Fig. 2. There was a correlation between the inhibitory effect of 8brcGMP on aggregation and that on TXB_2 formation in response to collagen.

Effect of 8brcGMP *on AA-metabolism stimulated by collagen*

Although 8brcGMP inhibited collagen-induced TXB_2 formation, this nucleotide little affected the metabolism of exogenous AA in rabbit platelets. Addition of AA (1 μg/ml at the final concentration) to platelet suspension increased TXB_2 level of the medium from 0.08 $\pm$ 0.03 to 10.4 $\pm$ 1.6 ng/10^8 platelets (N = 5). The conversion of exogenous AA to TXB_2 by platelets was not significantly altered by 1 mM 8brcGMP (TXB_2 levels was increased from 0.07 $\pm$ 0.02 to 8.7 $\pm$ 0.9 ng/10^8 platelets, N = 4). We therefore examined the effect of 8brcGMP on collagen-induced endogenous AA liberation using [^{14}C]AA-prelabeled platelets (Fig. 3). Collagen (1 μg/ml) caused AA release from platelets with a time course similar to that for TXB_2 formation. Even in the presence of indomethacin (2.5 μM), collagen could stimulate the AA liberation about 50% of control, whereas aggregation and TXB_2 formation were abolished (Fig. 1). 8brcGMP markedly inhibited the AA liberation by collagen either in the presence or absence of indomethacin (Fig. 3).

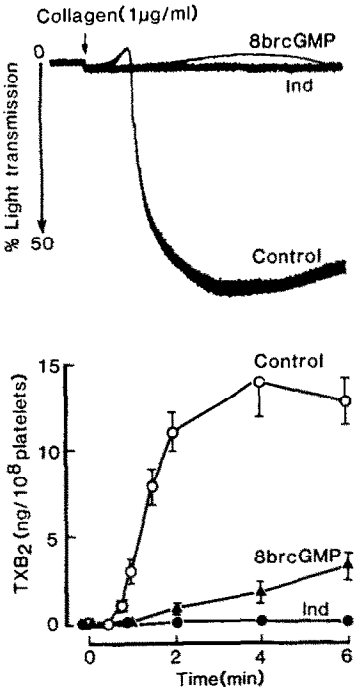

Fig. 1. Effect of 8brcGMP or indomethacin (Ind) on collagen-induced platelet aggregation (upper) and TXB_2 formation (lower). Samples of platelet suspension (1 ml) were preincubated for 3 min with saline (control), 8brcGMP (1 mM) or indomethacin (2.5 μM). After stimulation with collagen (1 μg/ml), aliquots (0.1 ml) were withdrawn at the indicated times for measurement of TXB_2. Values represent means ± SE of six observations.

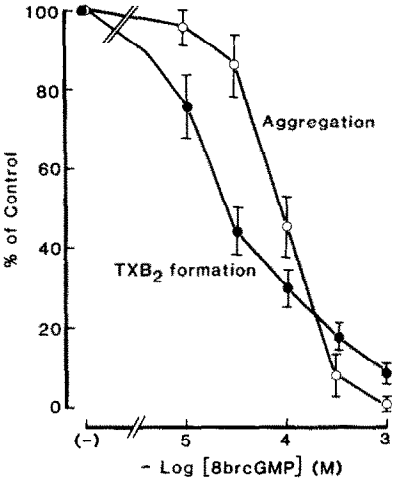

Fig. 2. Effect of various concentrations of 8brcGMP on collagen-induced platelet aggregation (○) and TXB_2 formation (●). After preincubation for 3 min with 8brcGMP at the concentration indicated on the abscissa, platelets were stimulated with collagen (1 μg/ml) for 4 min. Results were expressed as percentage of the control (without 8brcGMP) response. Values represent means ± SE of five observations.

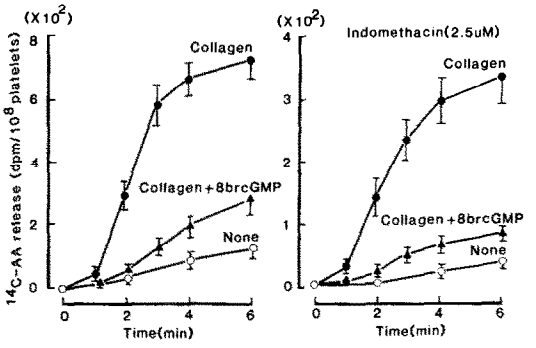

Fig. 3. Effect of 8brcGMP on collagen-induced AA liberation. Platelets prelabeled with [^{14}C]-AA were preincubated for 3 min with or without 8brcGMP (1 mM) and then stimulated with collagen (1 μg/ml). The reactions were performed in the presence (right) or absence (left) of indomethacin (2.5 μM). Values represent mean ± SE of six observations.

Role of extracellular Ca^{2+} in AA liberation by collagen

When the extracellular Ca^{2+} was omitted and EGTA was added at the final concentration of 0.2 mM, collagen induced neither platelet aggregation nor TXB_2 formation (Fig. 4). Addition of 2 mM Ca^{2+} to platelet suspension which had been incubated for 4 min with collagen in the Ca^{2+}-free medium, initiated platelet shape change and aggregation in parallel with TXB_2 formation. In the presence of 1 mM 8brcGMP, however, the platelets pretreated with collagen in Ca^{2+}-free medium hardly responded to the addition of Ca^{2+} (Fig. 4).

Phosphoinositide hydrolysis by collagen

The contribution of the phospholipase C pathway in the action of collagen was examined. Phospholipase C activation was evaluated by measurement of total inositol phosphates (IPs) production from [^{3}H]inositol-labeled platelets in the presence of 10 mM Li^+. Results are shown in Fig. 5. Collagen (1 μg/ml) failed to induce IPs production in Ca^{2+}-free medium. In the presence of 2 mM Ca^{2+}, collagen caused IPs accumulation about six-fold above the control levels. Collagen-induced IPs accumulation was inhibited about 80% by 1 mM 8brcGMP, and abolished by indomethacin (2.5 μM). These results suggest that phospholipase C activation in collagen-stimulated platelets is mediated by TXA_2, and collagen stimulates AA liberation in a manner independent of phospholipase C activation.

Effect of 8brcGMP on collagen-induced protein phosphorylation

In [^{32}P]orthophosphate-labeled platelets, collagen (1 μg/ml) stimulated phosphorylation of 20- and 40-kD proteins, which are the substrates for myosin light chain kinase [18] and protein kinase C [19], respectively (Fig. 6). The phosphorylation of 40 kD protein was stimulated after a lag period of 60 sec, similar to the AA liberation and TXB_2 formation (Fig. 1), and markedly inhibited by 2.5 μM indomethacin (Fig. 7). In contrast, the phosphorylation

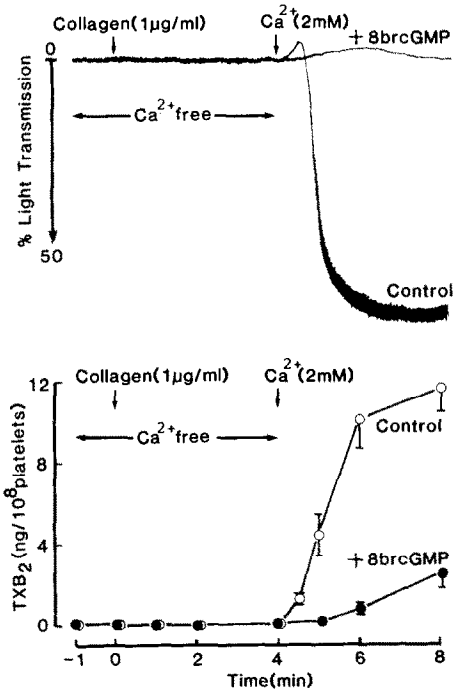

Fig. 4. Effect of removal of extracellular Ca^{2+} on collagen-induced platelet aggregation (upper) and TXB_2 formation (lower). Platelets suspended in a Ca^{2+}-free Hepes buffer containing 0.2 mM EGTA were stimulated with collagen (1 μg/ml) in the presence or absence of 8brcGMP (1 mM). $CaCl_2$ (2 mM) was added 4 min after the collagen. Values represent means ± SE of six observations.

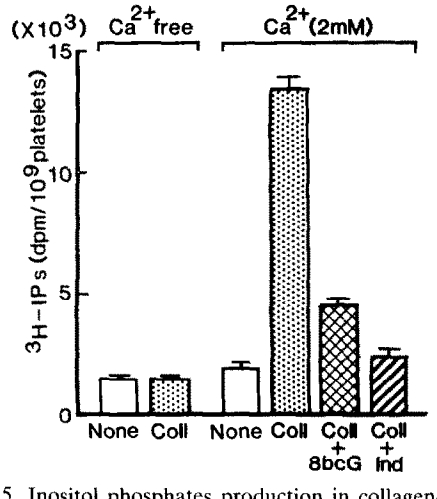

Fig. 5. Inositol phosphates production in collagen-stimulated platelets. Platelets prelabeled with [^{3}H]inositol were suspended in a Ca^{2+}-free (0.2 mM EGTA) or Ca^{2+} (2 mM)-containing Hepes buffer, and incubated for 10 min with LiCl (10 mM). After additional 3 min preincubation with saline (None), 1 mM 8brcGMP or 2.5 μM indomethacin (Ind), platelets were stimulated with 1 μg/ml collagen (Coll) for 5 min. Values represent means ± SE of five observations.

of 20-kD protein preceded the AA liberation and TXB_2 formation (observed as early as 30 sec), and hardly affected by indomethacin (Figs. 6 and 7). Preincubation with 8brcGMP (1 mM) for 3 min stimulated a 46-kD protein phosphorylation, and abolished collagen-induced both 20- and 40-kD protein phosphorylations (Figs. 6 and 7). Previously, the substrate of cyclic GMP-dependent protein kinase in human platelets was suggested to be identical with that of cyclic AMP-dependent protein kinase [7]. In rabbit platelets, however, the 46-kD protein phosphorylation appears to be selectively stimulated in a cyclic GMP-dependent manner, since dibutyryl cyclic AMP (dbcAMP, 1 mM) led to a significant phosphorylation of a 64-kD protein rather than that of 46-kD protein (Figs. 6 and 7). DbcAMP also abolished collagen-induced protein phosphorylations (Figs. 6 and 7) as well as AA liberation and aggregation in rabbit platelets (data not shown).

Role of Na^+–H^+ *exchange in collagen-induced AA liberation*

It has been recently suggested that an activation of Na^+–H^+ exchange at the plasma membrane plays an important role in epinephrine- and ADP-induced AA liberation in human platelets [20, 21]. Therefore, role of the Na^+–H^+ exchange in collagen-induced AA liberation was examined. Removal of extracellular Na^+ or increase in extracellular H^+ by decreasing extracellular pH from 7.4 to 6.8 has been reported to block the Na^+–H^+ exchange [20–22]. In washed rabbit platelets, collagen caused a normal aggregation in a Na^+-free (NaCl was replaced with *N*-methyl-D-glycamin) or low pH (6.8) medium (data not shown). Furthermore, collagen-induced AA liberation was also unaffected by the modification in extracellular Na^+ or pH (Fig. 8).

Effect of 8brcGMP *on* A-23187*-induced platelet activation*

Stimulation of [^{14}C]AA-prelabeled platelets with Ca^{2+} ionophore A23187 (0.5 μM) caused platelet aggregation and AA liberation. In contrast to the response to collagen, 8brcGMP (1 mM) had no effect on A23187-induced platelet aggregation and AA liberation (Fig. 9).

DISCUSSION

Cyclic GMP has been suggested to serve as an inhibitory mediator in the regulation of platelet functions [5–9, 15]. The present study reported here suggests that cyclic GMP has a negative regulatory effect on collagen-induced AA liberation in washed rabbit platelets. The results showed that (1) collagen at the concentration of 1 μg/ml induced platelet activation in a manner absolutely dependent upon TXA_2 formation; (2) collagen-induced aggregation and TXB_2 formation were inhibited by 8brcGMP; (3) there was a correlation between the inhibitory effect of 8brcGMP on TXB_2 formation and that on aggregation in response to collagen and (4) 8brcGMP did not affect the metabolic pathway of AA to TXA_2 in platelets but did inhibit the collagen-induced AA liberation from [^{14}C]AA-prelabeled platelets. These findings indicate that the inhibitory action of

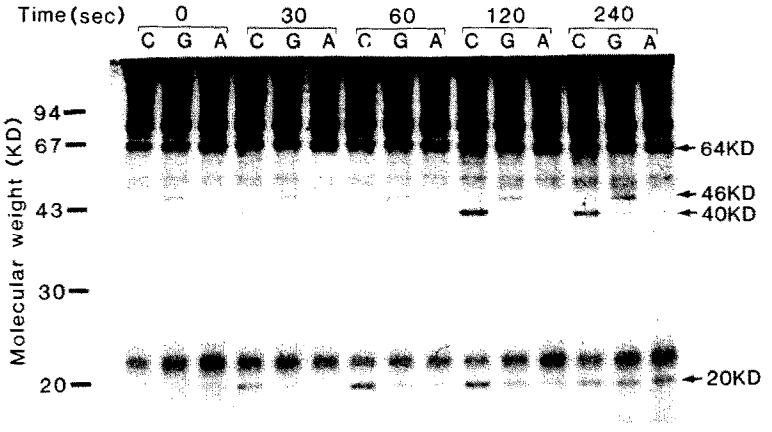

Fig. 6. Collagen-induced protein phosphorylation. ^{32}P-labeled platelets were preincubated at 37° for 3 min with saline (control; C), 1 mM 8brcGMP (G) or 1 mM dbcAMP (A). Platelets were then stimulated with collagen (1 μg/ml) for indicated times. Proteins of each sample were separated by SDS–PAGE and radioactive bands were localized by autoradiography which is shown in the figure. Two separated experiments gave the same results.

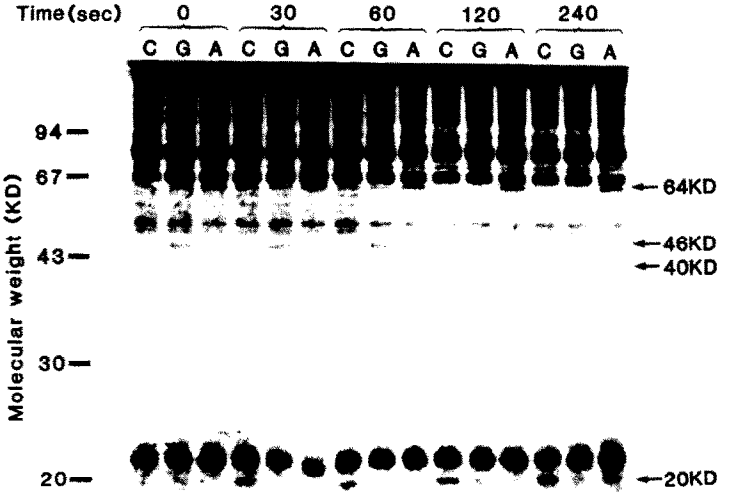

Fig. 7. Collagen-induced protein phosphorylation in the presence of indomethacin. ^{32}P-labeled platelets were treated with indomethacin (2.5 μM). Others are as in Fig. 6.

8brcGMP on collagen-induced platelet activation is due to the inhibition of AA liberation.

Previously, Rittenhouse and Allen [23] have suggested that the cyclooxygenase products formed at early period in response to collagen act as a positive feedback promoter for stimulation of AA liberation in human platelets. Similar mechanism may exist in the response of rabbit platelets to collagen, since a suppression of TXA_2 formation by indomethacin attenuated collagen-induced AA liberation by 50%. We have recently shown that 8brcGMP prevents TXA_2-mediated platelet activation [15]. These observations and the present results that collagen-induced TXB_2 formation was markedly inhibited, but not abolished by 8brcGMP, give rise to a possibility that cyclic GMP might impair the positive feedback effect of TXA_2 on AA liberation. However, collagen-induced AA liberation in indomethacin-treated platelets was also markedly inhibited by 8brcGMP. Thus, cyclic GMP appears to inhibit collagen-induced AA liberation through a mechanism other than its anti-TXA_2 activity.

The mechanism of collagen-induced AA liberation in platelets is not clearly understood. In platelets, three major pathways have been postulated for AA liberation; (1) direct action of phospholipase A_2 [24]; (2) diacylglycerol lipase following phospholipase C [25] and (3) phosphatidic acid-specific phospholipase A_2 following phospholipase C/diacylglycerol kinase [26]. Recent observations have provided evidence that cyclic GMP inhibits the activation of phosphatidylinositol-specific phospholipase C [7–9]. Namely 8brcGMP inhibits thrombin-stimulated production of diacylglycerol [7, 9] and inositol 1,4,5-triphosphate [9] which have been identified as second messengers for protein kinase C activation [27] and

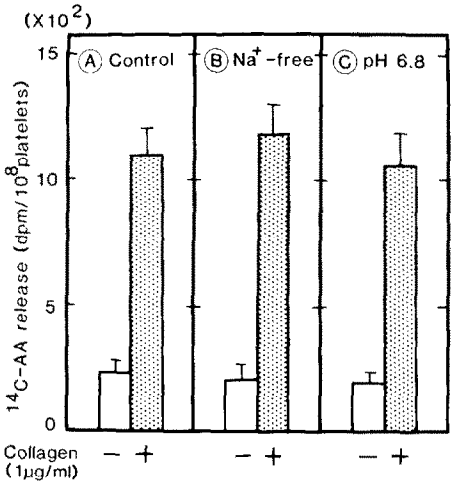

Fig. 8. Collagen-induced AA liberation in [^{14}C]AA-prelabeled platelets suspended in normal (A), Na^+-free (B) or low pH (C) Hepes buffer. Platelets were prepared as described under Materials and Methods. After 3 min preincubation, platelets were stimulated with collagen (1 μg/ml) for 4 min. Values represent means ± SE of four observations.

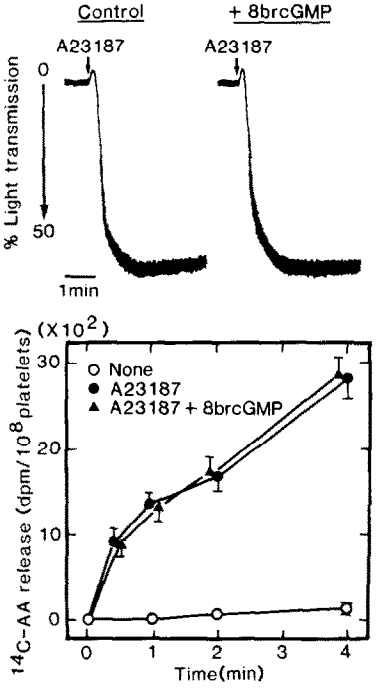

Fig. 9. Effect of 8brcGMP on A23187-induced platelet aggregation (upper) and AA liberation (lower). [^{14}C]AA-prelabeled platelets were stimulated with A23187 (0.5 μM) in the presence or absence of 8brcGMP (1 mM). Values represent mean ± SE of five observations.

mobilization of Ca^{2+} from intracellular stores [28], respectively. In addition, these second messenger molecules have been suggested to potentiate the AA liberation mediated by phospholipase A_2 [29, 30]. Collagen has been reported to induce platelet activation accompanied by phosphoinositide hydrolysis [14, 30]. It therefore seems possible that 8brcGMP might inhibit collagen-induced AA liberation as the result of inhibiting the activation of phospholipase C. The present study, however, provided evidence against this possibility by demonstrating that (1) indomethacin abolished collagen-induced IPs accumulation, but not AA liberation and (2) a 40-kD protein phosphorylation by protein kinase C in response to collagen was parallel with TXB_2 formation, and inhibited by indomethacin, indicating that AA liberation precedes phospholipase C activation. The present results are, however, apparently in conflict with recent observations by Watson *et al.* [31] who have shown an accumulation of inositol trisphosphate in indomethacin-treated human platelets stimulated with collagen. This difference may be due to the concentration of collagen used, since the concentration of collagen in their experiments was 10 times or more higher than that in the present study. We also observed that collagen at the concentration over 10 μg/ml caused a slight but significant accumulation of IPs even in the presence of indomethacin (unpublished study). These data suggest that at the concentration of collagen used in the present study (1 μg/ml), phospholipase C plays a minor role in the initiation of AA liberation from membrane phospholipids. The fact that collagen-induced IPs accumulation is abolished by removal of extracellular Ca^{2+} further supports the lack of direct effect of collagen on phospholipase C activation, because the activation of phosphoinositide-specific phospholipase C as a consequence of specific agonist-receptor interaction is independent of extracellular Ca^{2+} [10, 12–14]. Therefore, it is suggested that cyclic GMP inhibits AA liberation mediated by phospholipase A_2 activation in collagen-stimulated rabbit platelets.

Recent studies have demonstrated an importance of Na^+–H^+ exchange for AA liberation by phospholipase A_2 in response to ADP and epinephrine in human platelets [20, 21]. This mechanism, however, seems not to be involved in collagen-induced AA liberation in rabbit platelets, since the inhibition of Na^+–H^+ exchange by removal of extracellular Na^+ or by decreasing extracellular pH from 7.4 to 6.8, did not affect collagen-induced aggregation and AA liberation. In contrast, collagen could not cause any platelet response in the absence of extracellular Ca^{2+}, indicating that collagen-induced phospholipase A_2 activation in rabbit platelets is absolutely dependent upon the extracellular Ca^{2+}. Furthermore, collagen stimulated a 20-kD protein phosphorylation which is known to be mediated by a Ca^{2+}-dependent myosin light chain kinase [18]. In contrast to the phosphorylation of 40-kD protein, that of 20-kD protein in response to collagen preceded AA liberation and TXB_2 formation, and unaffected by indomethacin. Although the role of 20-kD protein phosphorylation in collagen-induced phospholipase A_2 activation is unclear at present, these data indicate that collagen causes an increase in the intracellular Ca^{2+} levels prior to the phospholipase A_2 activation. Since collagen has been reported to cause AA liberation without a detectable increase in the intracellular

Ca^{2+} levels in Fura 2-loaded human platelets [32], the amount of Ca^{2+} mobilized from the extracellular space may be small but sufficient for the Ca^{2+}-dependent phospholipase A_2 activation. The finding that 8brcGMP had no effect on Ca^{2+} ionophore A23187-induced platelet aggregation and AA liberation, indicates that cyclic GMP does not have a direct inhibitory effect on the processes subsequent to Ca^{2+} entry. Thus, it can be concluded that cyclic GMP may regulate the availability of extracellular Ca^{2+} which is responsible for phospholipase A_2 activation in collagen-stimulated platelets.

It is generally thought that cyclic nucleotides including cyclic GMP regulate cellular function through a stimulation of phosphorylation of the specific proteins. The present study demonstrates that 8brcGMP stimulates phosphorylation of a 46-kD protein in rabbit platelets. The 46-kD protein phosphorylation was also stimulated by nitroprusside (unpublished data), indicating that the 46-kD protein phosphorylation may be mediated by a cyclic GMP-dependent protein kinase. In human platelets, 8brcGMP and nitroprusside has been shown to enhance a 50-kD protein phosphorylation [7]. The characteristic of 46-kD protein, however, seems to be different from that of 50-kD protein found in human platelets, because the 50-kD protein is phosphorylated more significantly by dbcAMP than by 8brcGMP [33], whereas dbcAMP had little effect on the phosphorylation of 46-kD protein, but selectively stimulated that of a 64-kD protein in rabbit platelets. Thus, further investigation on the role of the 46-kD protein in platelet function are important for understanding the precise mechanism of action of cyclic GMP in rabbit platelets.

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