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PARALLEL INHIBITION OF PLATELET-ACTIVATING FACTOR-INDUCED PROTEIN PHOSPHORYLATION AND SEROTONIN RELEASE BY K-252a, A NEW INHIBITOR OF PROTEIN KINASES, IN RABBIT PLATELETS

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Abstract—K-252a, (8*R**,9*S**,11*S**)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadi benzo[*a*,*g*]cycloocta[*c*,*d*,*e*]trinden-1-one, an indole carbazol compound isolated from microbial origin, potently inhibits protein kinase C in partially purified enzyme and intact platelets. We examined the effects of this compound on platelet-activating factor [1-*O*-alkyl-*α*-acetyl-sn-glycero-phosphocholine (AGEPC)] induced protein phosphorylation, serotonin release and a rise in intracellular free calcium using washed rabbit platelets. In Ca^{2+}-containing medium (1 mM $CaCl_2$), AGEPC at 10^{-10} and 10^{-9} M markedly phosphorylated two proteins having molecular weights of 40,000 daltons (40 K protein) and 20,000 daltons (20 K protein) and evoked a marked rise in cytosolic free calcium. K-252a at 3 and 10 μM caused a concentration-dependent inhibition in the 20 K protein phosphorylation but caused only slight inhibition in the 40 K protein phosphorylation. K-252a inhibited the basal phosphorylation of 20 K protein obtained in non-stimulated platelets, and caused no significant alteration in the rise of intracellular free calcium evoked by AGEPC. It can be considered, from this evidence, that K-252a may act directly on myosin light chain kinase, resulting in the inhibition of 20 K protein phosphorylation. In Ca^{2+}-free medium [1 mM ethylene glycol-bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)], AGEPC at 10^{-8} M predominantly phosphorylated 40K protein, although phosphorylation of 20K protein and cytosolic free calcium were increased slightly. K-252a at 1–10 μM caused a concentration-dependent inhibition in the 40K protein phosphorylation. These results indicate that K-252a functions as an inhibitor of both protein kinase C and myosin light chain kinase in rabbit platelets. In AGEPC-stimulated platelets, the inhibition of 20K protein phosphorylation in Ca^{2+}-containing medium and of 40K protein phosphorylation in Ca^{2+}-free medium was closely correlated with the inhibition of serotonin release by K-252a. These results strongly suggest that the phosphorylation of these two proteins may be a prerequisite for serotonin release in AGEPC-stimulated platelets.

K-252a, (8*R**,9*S**,11*S**)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadi benzo[*a*,*g*]cycloocta-[*c*,*d*,*e*]trinden-1-one, a metabolite isolated from *Nocardiopsis* sp. has been found to inhibit potently protein kinase C [1]. Many lines of evidence have revealed that protein kinase C has a critical role in signal transduction in various cell types including platelets.

Platelet-activating factor, 1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine (AGEPC)†, is a potent phospholipid mediator able to promote platelet activation [2]. AGEPC may act on platelets through specific binding to a putative receptor [3–5]. AGEPC induces a rapid breakdown of inositol phospholipids which results in the release of diacylglycerol. The diacylglycerol appears to function as an important second messenger in the platelets through the activation of protein kinase C, which has been demonstrated to phosphorylate 40,000 dalton protein (40K protein) [6–8]. Furthermore, AGEPC also elevates cytosolic free calcium ($[Ca^{2+}]_i$) [9–11], and this might lead to activation of Ca^{2+}-calmodulin dependent myosin light chain kinase, resulting in phosphorylation of 20,000 dalton protein (20K protein) which has been characterized as myosin light chain [12, 13]. These two pathways are hypothesized to function synergistically for full-activation of dense granule secretion [14, 15].

In a previous paper [16], we demonstrated that K-252a inhibits 40K protein phosphorylation induced by a direct activator of protein kinase C, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), in intact platelets and proposed that the 40K protein phosphorylation may play a causative role in dense granule secretion induced by TPA. Effects of this compound on receptor-mediated platelet responses, however, have not yet been reported. Therefore, we examined the effects of K-252a on protein phosphorylation, serotonin release, cytosolic free calcium and production of arachidonate metabolites in AGEPC-

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† Abbreviations: AGEPC, 1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine; EGTA, ethylene glycol-bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; HHT, hydroxy-heptadecatrienoic acid; TXB_2, thromboxane B_2; 12-HETE, 12-hydroxy-eicosatetraenoic acid; and SDS, sodium dodecyl sulfate.

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stimulated platelets. The present paper shows that K-252a inhibited the phosphorylation of both 40K and 20K proteins and that the inhibition of these two protein phosphorylations may be a possible mechanism for the inhibition of serotonin release.

MATERIALS AND METHODS

Preparation of platelets. Blood anticoagulated with acid-citrate-dextrose was obtained from male rabbits and centrifuged for the separation of platelet-rich plasma (PRP). For analysis of serotonin release or arachidonic acid metabolism, PRP was incubated with [2-^{14}C]serotonin (0.2 μCi/10 ml PRP) or [2-^{14}C]arachidonic acid (1 μCi/10 ml PRP), respectively, for 60 min at 37°. Then platelets were pelleted from PRP by centrifugation at 1600 *g* for 10 min. The precipitated platelets were washed twice and suspended in Tyrode's solution containing 138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 2.0 mM $MgCl_2$ and 5.5 mM glucose. For analysis of protein phosphorylation or for measurement of concentration of cytosolic free calcium ($[Ca^{2+}]_i$), the platelets were labeled with $^{32}P_i$ according to the method of Shaw and Lyons [7], or were incubated with 5 μM quin 2 acetoxymethyl ester according to the method of Kawahara *et al.* [17] respectively. The platelets labeled with $^{32}P_i$ or loaded with quin 2 were finally suspended in the same Tyrode's solution at 2×10^9 cells/ml or 2×10^8 cells/ml respectively. After stabilization for 30 min, 1 mM $CaCl_2$ or 1 mM EGTA was added.

Measurement of serotonin release and protein phosphorylation. A suspension of platelets that were prelabeled with [2-^{14}C]serotonin or $^{32}P_i$ was preincubated with vehicle or K-252a solution at 37° for 3 min, and then activated by the addition of AGEPC. For measuring serotonin release, the reaction was terminated by the addition of ice-cold 0.1 mM formaldehyde and 5 mM EDTA. The mixture was centrifuged for 3 min at 1000 *g*, and the radioactivity in the supernatant fraction was counted with liquid scintillation counter. For analysis of phosphoproteins, the reaction was terminated by the addition of 1.1% SDS containing 5% mercaptoethanol. The mixture was boiled at 100° for 2 min and subjected to SDS-polyacrylamide gel electrophoresis as described by Laemmli [18]. The gel was then stained with Coomassie brilliant blue, dried, and exposed to Fuji X-ray film to prepare the autoradiogram. The relative intensity of each band was quantitated by densitometric tracing of the autoradiogram at 430 nm using a Shimazu dual wavelength chromatogram scanner, model CS-930.

Measurement of cytosolic free calcium. A suspension of quin 2-loaded platelets was stirred continuously with a Teflon-covered magnetic follower in a Hitachi F-3000 fluorescence spectrophotometer at 37°. $[Ca^{2+}]_i$ was measured by quin 2 fluorescence (excitation 339 ± 5 nm; emission 500 ± 10 nm) and calculated as described [9].

Arachidonic acid metabolism. A suspension of platelets prelabeled with [^{14}C]arachidonic acid was preincubated with vehicle or K252a solution for 5 min at 37° and then activated by the addition of AGEPC. After a further 5-min incubation, the metabolites were extracted by the method of Yahn and Feinstein [19]. The extract was evaporated under N_2 vapor, and the residue was redissolved with acetone. Arachidonic acid metabolites were separated by thin-layer chromatography on a silica gel plate (Whatman, LK6-D) in a solvent system consisting of the upper phase of a mixture of ethyl acetate–2,2,4-trimethylpentane–acetic acid–water (110:50:20:100) [20]. The lipids were visualized by autoradiography and counted by liquid scintillation.

Materials. K252a was isolated from culture broth of *Nocardiopsis* sp. as reported previously [1]. Synthetic platelet-activating factor, AGEPC, was purchased from Sigma. Quin 2 acetoxymethyl ester was a product of Dojin. [2-^{14}C]Serotonin, [2-^{14}C]arachidonic acid and $^{32}P_i$ were obtained from New England Nuclear. Other chemicals were obtained from commercial sources.

RESULTS

Effect on serotonin release. AGEPC at 10^{-10} and 10^{-9} M evoked a concentration-dependent release of serotonin in platelets suspended in medium containing 1 mM $CaCl_2$ (Ca^{2+}-containing medium). The mean rates of the serotonin release were 26.4 and 65.2% at 10^{-10} and 10^{-9} M AGEPC respectively. When the $CaCl_2$ in the medium was replaced by 1 mM EGTA to deplete extracellular calcium (Ca^{2+}-free medium), serotonin release reaction induced by AGEPC became less marked. The mean rates of the release were 17.0 and 32.0% at 10^{-9} M and 10^{-8} M AGEPC respectively.

The effects of K-252a on AGEPC-induced serotonin release are summarized in Fig. 1. K-252a suppressed serotonin release in a concentration-dependent manner in both Ca^{2+}-containing and Ca^{2+}-free media. The IC_{50} values of K-252a were 1.6 and 4.6 μM at 10^{-10} and 10^{-9} M AGEPC in Ca^{2+}-containing medium, and 1.7 and 3.9 μM at 10^{-9} M and 10^{-8} M AGEPC in Ca^{2+}-free medium respectively.

Effects on protein phosphorylation. The effects of K-252a on AGEPC-induced protein phosphorylation in platelets were examined in both Ca^{2+}-containing and Ca^{2+}-free media. The summarized data are shown in Tables 1 and 2. Several papers [6, 9, 21] described induction by AGEPC of a rapid protein phosphorylation which reaches maximum level within 30 sec after the stimulation. Therefore, the drug effects were assessed at 30 sec after the addition of AGEPC.

In Ca^{2+}-containing medium, AGEPC at 10^{-10} M and 10^{-9} M phosphorylated both 20K and 40K protein in a concentration-dependent manner. K-252a inhibited the 20K protein phosphorylation at 3 and 10 μM. Almost complete inhibition was obtained at 10 μM K-252a. The concentration range of K-252a that inhibited the 20K protein phosphorylation was nearly equal to that which inhibited serotonin release. On the other hand, the 40K protein phosphorylation was hardly affected by K-252a at 1–3 μM, although it was inhibited slightly by 10 μM K-252a, (Table 1).

In Ca^{2+}-free medium, AGEPC at 10^{-9} and 10^{-8} M phosphorylated 40K protein markedly but not 20K

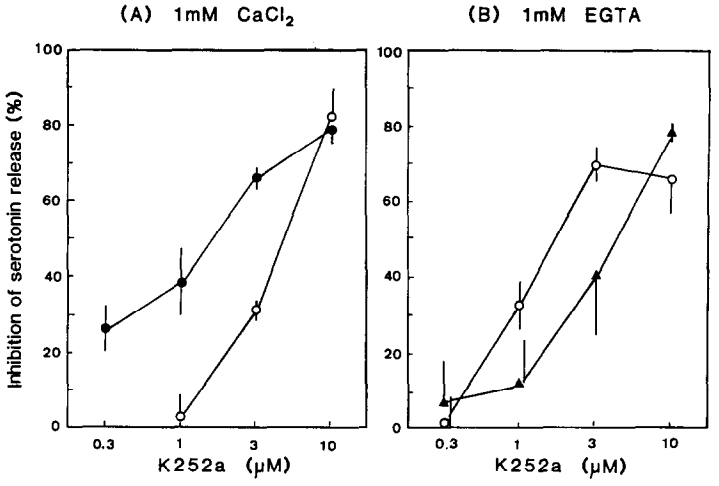

Fig. 1. Inhibition of platelet-activating factor-induced serotonin release from rabbit platelets by K-252a. Platelets (5×10^8 cells) suspended in medium containing 1 mM $CaCl_2$ (A) or 1 mM EGTA (B) were preincubated with K-252a for 3 min before the addition of 10^{-10} M (●), 10^{-9} M (○) or 10^{-8} M (▲) AGEPC. Each point represents the mean ± SEM of three separate experiments with duplicate determinations.

Table 1. Effects of K-252a on protein phosphorylation induced by platelet-activating factor (AGEPC) in rabbit platelets in Ca^{2+}-containing medium

Treatment	Relative rate of phosphorylation (%)			
	AGEPC (10^{-10} M)		AGEPC (10^{-9} M)	
	20 K	40 K	20 K	40 K
Basal (without AGEPC)	100	100	100	100
Control (with AGEPC)	208 ± 16	163 ± 20	286 ± 28	304 ± 18
K-252a 1 μM	218 ± 36	170 ± 20	273 ± 27	295 ± 17
3 μm	178 ± 52	163 ± 29	247 ± 18	292 ± 11
10 μM	87 ± 3	153 ± 31	97 ± 15	204 ± 25

^{32}Pi-labeled platelets suspended in the medium containing 1 mM $CaCl_2$ were preincubated with K-252a for 3 min before the addition of AGEPC. After a 30-sec incubation with AGEPC, protein phosphorylation was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Each value represents the mean ± SE of three separate experiments.

Table 2. Effects of K-252a on protein phosphorylation induced by platelet-activating factor (AGEPC) in rabbit platelets in Ca^{2+}-free medium

Treatment	Relative rate of phosphorylation (%)			
	AGEPC (10^{-9} M)		AGEPC (10^{-8} M)	
	20 K	40 K	20 K	40 K
Basal (without AGEPC)	100	100	100	100
Control (with AGEPC)	140 ± 7	191 ± 29	157 ± 19	256 ± 36
K-252a 1 μM	136 ± 5	183 ± 33	118 ± 34	191 ± 33
3 μM	87 ± 10	117 ± 13	98 ± 36	145 ± 34
10 μM	63 ± 3	82 ± 2	71 ± 37	92 ± 16

$^{32}P_i$-labeled platelets suspended in the Ca^{2+}-free medium with 1 mM EGTA were used. Other details were the same as in Table 1. Each value represents the mean ± SE of three separate experiments.

protein. The 40K protein phosphorylation was clearly inhibited by K252a at 1–10 μM in a concentration-dependent manner, in contrast to the Ca^{2+}-containing medium. The inhibition of 40K protein phosphorylation was closely correlated with the inhibition of serotonin release. Although the increase of 20K protein phosphorylation obtained after AGEPC stimulation was small, it was suppressed by K-252a, and the rate of the phosphorylation in samples treated with 10 μM K-252a was lower than that in non-stimulated platelets (basal phosphorylation). Next, the effect of K-252a alone on the basal phosphorylation of 20K protein was examined. As shown in Fig. 2, K-252a inhibited the basal phosphorylation of 20K protein in a concentration-dependent manner.

Effects on cytosolic free calcium. We examined the effects of K-252a on the rise in $[Ca^{2+}]_i$ in response to AGEPC, in order to elucidate whether the inhibition of 20K protein phosphorylation is due to a suppression of the $[Ca^{2+}]_i$ level.

Representative tracings of quin 2 fluorescence are shown in Fig. 3. In Ca^{2+}-containing medium, AGEPC at 10^{-10} M markedly elevated $[Ca^{2+}]_i$ from the resting level (about 100 nM) to approximately 800 nM. In Ca^{2+}-free medium, the resting level of $[Ca^{2+}]_i$ was about half of that in Ca^{2+}-containing medium, and $[Ca^{2+}]_i$ did not exceed the resting level in Ca^{2+}-containing medium even after stimulation by 10^{-8} M AGEPC.

K-252a at 3 μM and lower hardly affected the elevation of $[Ca^{2+}]_i$ induced by AGEPC in either Ca^{2+}-containing or Ca^{2+}-free medium. Since K-252a *per se* at 10 μM and more has a potent fluorescence and this disturbed the measurement of quin 2 fluorescence, the effect of a higher concentration of K-252a could not be determined.

Effects on production of arachidonate metabolites. It is known that AGEPC stimulates the formation of arachidonate metabolites which is inhibited by the so-called calmodulin antagonist trifluoperazine [22]. K-252a is also known to possess calmodulin antagonistic action with activity superior to that of trifluoperazine [1]. Therefore, the effect of K-252a on the production of arachidonate metabolites was examined. As shown in Fig. 4, K252a caused no significant change in cyclooxygenase products, TXB_2 + HHT, or a lipoxygenase product, 12-HETE, induced by 10^{-9} M AGEPC in Ca^{2+}-containing medium.

DISCUSSION

We recently showed that K-252a elicits the inhibition of protein kinase C in TPA-stimulated platelets [16]. In the present study, we found that K-252a also served as an inhibitor of myosin light chain kinase in intact platelets. AGEPC markedly elevated the phosphorylation of 20K protein of platelets, accompanied by a rise in $[Ca^{2+}]_i$ in Ca^{2+}-containing medium. The elevated level of $[Ca^{2+}]_i$ may be higher than that required to activate Ca^{2+}-calmodulin-dependent myosin light chain kinase [23]. In contrast, in Ca^{2+}-free medium, only a slight increase of 20K protein phosphorylation was seen and $[Ca^{2+}]_i$ was not elevated over the resting level obtained in Ca^{2+}-containing medium, even after activation by AGEPC. It can be concluded from these data

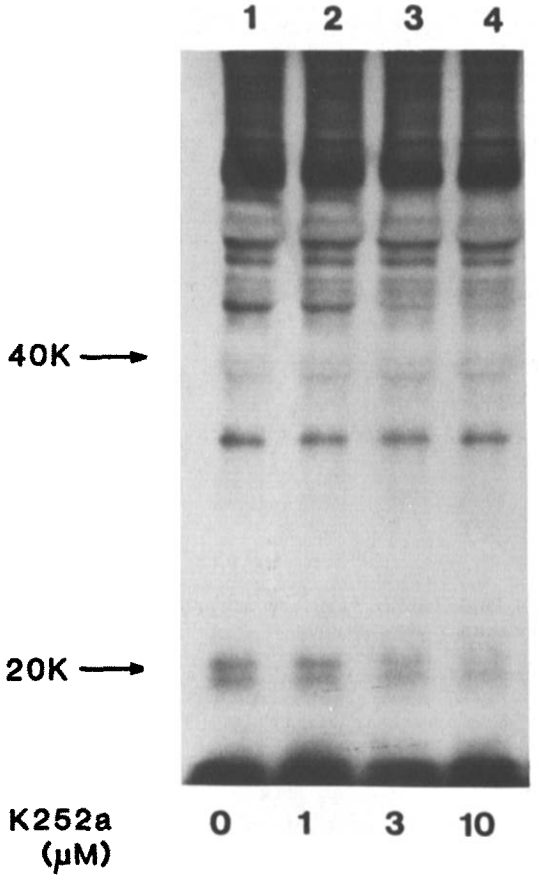

Fig. 2. Effect of K-252a on basal phosphorylation of 20K protein in non-stimulated rabbit platelets. ^{32}Pi-labeled platelets suspended in the Ca^{2+}-free medium were incubated with K-252a for 3.5 min. Protein phosphorylation was analyzed as described under Materials and Methods.

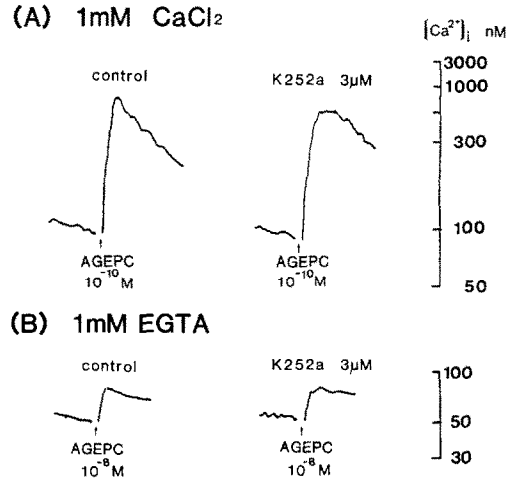

Fig. 3. Effects of K-252a on the rise in cytosolic free calcium level in response to platelet-activating factor in rabbit platelets. Platelets loaded with quin 2 were preincubated with K-252a for 3 min before the addition of AGEPC.

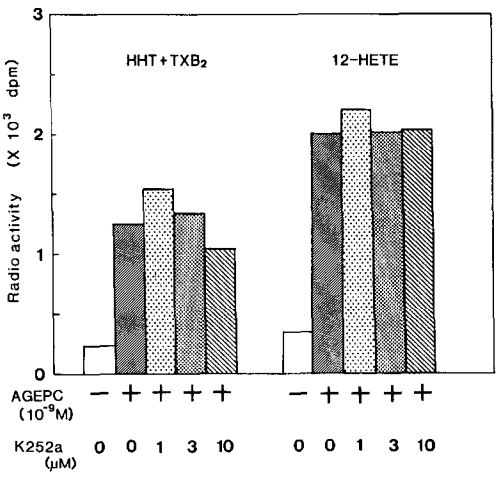

Fig. 4. Effects of K-252a on the production of arachidonate metabolites induced by platelet-activating factor in rabbit platelets. Platelets prelabeled with [^{14}C]arachidonic acid were preincubated with K252a for 3 min before the addition of AGEPC (10^{-9} M). After a further 5 min of incubation, arachidonate metabolites were analyzed as described in Materials and Methods. Each value represents the mean of duplicate determinations. Similar data were obtained in three experiments. Abbreviations: HHT, hydroxy-heptadecatrienoic acid; TXB_2, thromboxane B_2; and 12-HETE, 12-hydroxy-eicosatetraenoic acid.

that the AGEPC-induced increase of 20K protein phosphorylation was dependent on the rise in $[Ca^{2+}]_i$, and that Ca^{2+}-calmodulin-dependent myosin light chain kinase was responsible for this reaction. K-252a inhibited the AGEPC-stimulated phosphorylation of 20K protein without affecting the rise in $[Ca^{2+}]_i$. This indicates that K-252a acted on the process after the elevation of $[Ca^{2+}]_i$. In addition, K-252a also inhibited the basal phosphorylation of 20K protein which occurred at the resting level of $[Ca^{2+}]_i$ in non-stimulated platelets. Hallam *et al.* [24] showed the existence of such myosin phosphorylation that may be controlled by the Ca^{2+}-calmodulin-independent pathway. These facts suggest that K-252a inhibited myosin phosphorylation in a Ca^{2+}-calmodulin-independent way. Furthermore, we found that K-252a directly inhibited smooth muscle myosin light chain kinase from chicken gizzard in competition with ATP (manuscript in preparation). This evidence demonstrates that K-252a directly interacts with myosin light chain kinase, resulting in the inhibition of myosin light chain phosphorylation in intact platelets.

As previously reported [6, 9, 19], a marked phosphorylation of 40K protein is induced by AGEPC in both Ca^{2+}-containing and Ca^{2+}-free media. K-252a showed a clear inhibition of the AGEPC-induced 40K protein phosphorylation only in Ca^{2+}-free medium. Since K-252a can inhibit 40K protein phosphorylation induced by TPA even in Ca^{2+}-containing medium [16], and TPA is known to cause no rise in $[Ca^{2+}]_i$ [10], we also confirmed in our preparation), it seems that the inhibitory effect of K-252a on 40K protein phosphorylation is reversed by the rise in $[Ca^{2+}]_i$. From kinetic analysis, the inhibitory action of K-252a on protein kinase C partially purified from rat brain is not reversed by calcium [25]. Therefore, the reversal observed in intact cells may not be due to competition with calcium on the enzyme. The mechanism for the reversal, however, remains to be elucidated.

Thus, it appears that K-252a functions as an inhibitor of myosin light chain kinase or of protein kinase C with or without elevation of $[Ca^{2+}]_i$ respectively.

There have been several reports which demonstrate that activation of Ca^{2+}-calmodulin-dependent myosin light chain kinase and protein kinase C are correlated with platelet dense granule secretion induced by several stimuli including platelet-activating factor [6, 8, 12, 13, 26]. A cause–effect relationship, however, was not demonstrated. Application of specific inhibitors of these enzymes may provide some clues in elucidating a cause–effect relationship.

AGEPC evoked serotonin release in parallel with phosphorylation of 20K and 40K proteins in Ca^{2+}-containing medium. K-252a concomitantly inhibited the serotonin release and 20K protein phosphorylation. It had been reported that so-called calmodulin antagonists, such as trifluoperazine and W-7, show similar and parallel inhibition of receptor-mediated dense granule secretion and 20K protein phosphorylation [27, 28]. These agents, however, also inhibit 40K protein phosphorylation [28] and arachidonic acid release from membrane phospholipids [29–31]. It is known that dense granule secretion evoked by AGEPC is roughly halved by blockade of cyclooxygenase [11]. Therefore, these actions are considered as possible candidates for the inhibition of dense granule secretion. Unlike these agents, K-252a inhibited serotonin release without significant alterations in 40K protein phosphorylation and production of arachidonate metabolites. Therefore, it can be concluded that K-252a inhibits serotonin release mainly due to the inhibition of 20K protein phosphorylation. This evidence strongly suggests that 20K protein phosphorylation is a prerequisite to initiation of receptor-mediated dense granule secretion in Ca^{2+}-containing medium.

In Ca^{2+}-free medium, AGEPC released small but significant amounts of serotonin. After stimulation by AGEPC the increase of 20K protein phosphorylation was fairly small, and the $[Ca^{2+}]_i$ level was not high enough to activate Ca^{2+}-calmodulin-dependent myosin light chain kinase [23]. Therefore, it can be considered that the AGEPC-induced release obtained in Ca^{2+}-free medium was independent of the Ca^{2+}-calmodulin pathway. Such Ca^{2+}-independent dense granule secretion has also been shown in platelets activated by thrombin, collagen and TPA [32]. A rapid and marked phosphorylation of 40K protein occurred in parallel with the Ca^{2+}-independent serotonin release. These two responses were inhibited by K-252a at equal ranges of concentrations. These results support the suggestion that 40K protein phosphorylation may be one of the prerequisites for initiation of receptor-mediated dense granule secretion, as proposed before [6, 8]. However, since little is known about the function of the 40K protein that is phosphorylated in response

to stimuli, the real interrelationship between 40K protein phosphorylation and dense granule secretion remains to be clarified.

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