Vol. 144, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1987 Pages 35-40

K252a, A NEW INHIBITOR OF PROTEIN KINASE C, CONCOMITANTLY INHIBITS 40K PROTEIN PHOSPHORYLATION AND SEROTONIN SECRETION IN A PHORBOL ESTER-STIMULATED PLATELETS

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Received December 17, 1986

SUMMARY. K252a isolated from microbial origin was found to potently inhibit protein kinase C in vitro (1). This agent inhibits phosphorylation of 40,000 dalton protein(40K protein) induced by 12-O-tetradecanoylphorbol-13-acetate(TPA) in intact rabbit platelets. This indicates that K252a exhibits the inhibition of protein kinase C in intact cells. The serotonin secretion induced by TPA was inhibited by K252a at nearly equal concentrations required to inhibit the phosphorylation of 40K protein. This provides the evidence to support the cause-effect relationship between the protein phosphorylation and the secretion in TPA-stimulated platelets.

Many lines of evidence revealed that protein kinase C has a critical role in signal transduction in various cell types. In platelets, agonist-receptor interaction at the plasma membrane induces a rapid breakdown of phosphoinositides which results in the release of diacylglycerol. The diacylglycerol appears to function as an important second messenger in the platelet through the activation of protein kinase C, which has been demonstrated to phosphorylate 40,000 dalton protein (40K protein) (2,3). 12-O-Tetradecanoylphorbol-13-acetate (TPA) can substitute for the diacylglycerol and activate protein kinase C resulting in the phosphorylation of 40K protein (3). TPA has been known to induce dense granule secretion without an elevation of cytoplasmic free calcium (4).

Kase et al. recently isolated K252a from culture broth of Nocardiopsis sp. and found this compound to be a potent inhibitor of protein kinase C in vitro (1).

Abbreviations: TPA, 12-O-tetradecanoylphorbol-13-acetate, EGTA, ethyleneglycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid.

0006-291X/87 $1.50

In this study, we examined the effects of K252a on phosphorylation of 40K protein and serotonin secretion in TPA-stimulated platelets and showed that the inhibition of the phosphorylation correlated closely with the inhibition of the secretion.

MATERIALS AND METHODS

Blood anticoagulated with acid-citrate-dextrose was obtained from male rabbits and centrifuged for the separetion of platelet-rich plasma (PRP). For analysis of serotonin secretion, PRP was incubated with [2-^{14}C] serotonin (0.2 μCi/10ml PRP) for 60 min at 37°C. Then platelets were pelleted from PRP by centrifugation at 600xg for 10 min. The precipitated platelets were washed twice and suspended in Tyrode's solution containing 138 mM NaCl, 2.7mM KCl, 12 mM $NaHCO_3$, 0.3mM NaH_2PO_4, 2.0mM $MgCl_2$ and 5.5mM glucose. For analysis of protein phosphorylation, the platelets were labelled with ^{32}Pi according to the method of Lyons et al (5), and finally suspended in the same solution. After stabilization for 30 min, 1 mM $CaCl_2$ or 1 mM EGTA was added.

A suspension of platelets which was prelabelled with ^{32}Pi or [2-^{14}C] serotonin was preincubated with vehicle or K252a solution at 37°C for 3 min, then stimulated by an addition of TPA (10^{-7}M). For analysis of phosphoproteins, the reaction was terminated by an addition of 1.1% SDS containing 5% mercaptoethanol. The mixture was boiled at 100°C for 2 min and subjected to SDS-polyacrylamide gel electrophoresis as described by Laemmli (6).

The gel was then stained with coomassie brilliant blue, dried and exposed to Fuji Xray film to prepare autoradiogram. The relative intensity of each band was quantitated by densitometric tracing of the autoradiogram at 430 nm using a Shimadzu dual wave length chromatogram scanner, Model CS-430. For measuring of serotonine secretion, the mixture was centrifuged for 3 min at 1000xg, and the radioactivity in the supernatant was counted with liquid scintillation counter.

[2-^{14}C]serotonin binoxalate and ^{32}Pi were purchased from New England Nuclear. TPA was a product of Sigma. Other chemicals were obtained from commercial source.

RESULTS AND DISCUSSION

As had been reported (3), when rabbit platelets were activated by 10^{-7}M TPA, phosphorylation of 40K protein was markedly elevated both in the presence and absence of extracellular calcium. On the other hand, no detectable increase was observed in phosphorylation of 20,000 dalton protein (20K protein) which had been identified as myosin light chain. In the absence of extracellular calcium, K252a suppressed the phosphorylation of 40K protein in a concentration-dependent fashion. This agent below 3 μM also showed the simmilar inhibition in the presence of extracellular calcium (Fig. 1). Since 40K protein in platelets appears to be a substrate of protein kinase C (3,7) and TPA induces phosphorylation of this protein

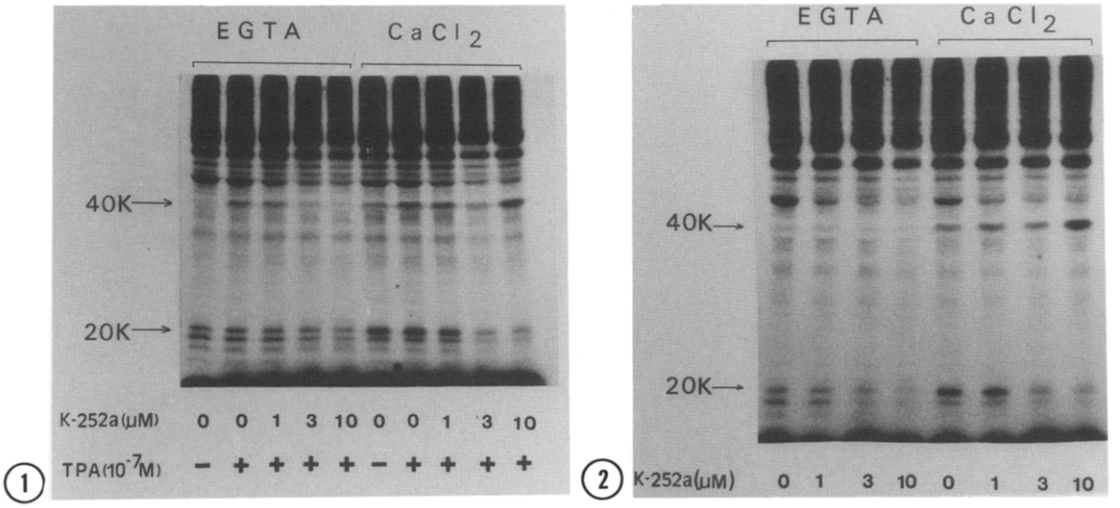

Fig.1. Inhibition by a protein kinase C inhibitor, K252a of 40K protein phosphorylation induced by TPA in rabbit platelets. The ^{32}Pi-labelled platelets were preincubated for 3 min at 37°C with or without K252a and then stimulated with 10^{-7}M TPA in the presence of 1mM EGTA or 1mM $CaCl_2$ for 2 min. Protein phosphorylation was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Fig.2. Effects of K252a on basal protein phosphorylation in rabbit platelets. The ^{32}Pi-labelled platelets were incubated with or without K252a for 5 min at 37°C in the presence of 1mM EGTA or 1mM $CaCl_2$. Protein phosphorylation was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

by direct activation of protein kinase C, it can be concluded that K252a exerts the inhibition of protein kinase C in intact cells in compatible with in in vitro enzyme preparation. When concentration of K252a increased to a level of 10 μM in the presence of $CaCl_2$, the agent apparently failed to inhibit 40K protein phosphorylation (Fig.1). In order to explore the cause of the lack of the inhibition, the effects of K252a alone on 40K protein phosphorylation were examined in non-stimulated platelets. As shown in Fig. 2, while the intensity of the phosphorylated band of 40K protein was not altered by K252a at 1-10 μM or below 3 μM in the presence of EGTA or $CaCl_2$, respectively, it was markedly strengthened by 10 μM K252a in the presence of $CaCl_2$. This reveals that 10 μM K252a in combination with extracellular calcium induces 40K protein phosphorylation. It seemed, from these results, that the apparent lack of inhibition of 40K protein phosphorylation by 10 μM K252a in the presence of $CaCl_2$ is not due to the

failure of protein kinase C inhibition, but due to the compensation by the increase of 40K protein phosphorylation induced by K252a per se. However, it remains to be clarified by what mechanism the agent induces the 40K protein phosphorylation.

TPA has been known to induce a slow rate of secretion of dense granule constituents such as ATP and serotonin (4). Different effects of inhibitors of protein kinase C on the TPA-induced secretion were reported. Phospholipid-interacting agents such as local anesthetics and trifluoperazine have been known to inhibit protein kinase C by competing with phosphatidylserine, an activating factor of this enzyme (8,9). Tetracaine which belongs to this class of inhibitors concomitantly inhibits serotonin secretion and 40K protein phosphorylation (3). Some other phospholipid-interacting agents also inhibit the dense granule secretion (10). Hidaka et al. discovered another class of inhibitor, H-7 which may inhibit protein kinase C by competing with ATP (11). Inagaki et at (12) have reported that this agent potentiates the serotonin secretion, and they proposed that the potentiation may be due to the inhibition of TPA-induced 20K protein phosphorylation which plays an inhibitory role in the secretion. Thus, the role of 40K protein in TPA-induced serotonin secretion has remained to be elucidated. Then, we examined the effect of K252a on serotonin secretion induced by TPA.

When platelets were stimulated by 10^{-7}M TPA, a fairly amount of serotonin was released into the medium. The mean secretion rate at 5 min after the addition of TPA was 35.4 ± 3.9% or 28.4 ± 3.9%, in the presence of EGTA or $CaCl_2$, respectively. K252a inhibited the TPA-induced serotonin secretion in a concentration-dependent fashion (Fig.3). This inhibition was closely correlated with the inhibition of 40K protein phosphorylation thoughout all concentrations of K252a except 10 μM K252a in the presence of $CaCl_2$. This exception may be due to the increase of 40K protein phosphorylation by K252a per se as described above.

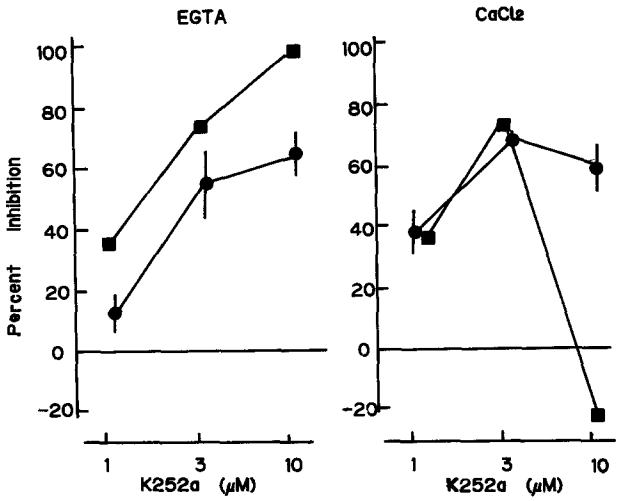

Fig.3. Parallel inhibition by K252a of serotonin secretion and 40K protein phosphorylation in TPA-stimulated platelets. The [2-^{14}C] serotonin-labelled platelets were preincubated with or without K252a for 3 min at 37°C and then stimulated with 10^{-7}M TPA in the presence of 1mM EGTA or 1mM $CaCl_2$ for 5 min. Serotonin secretion was quantitated as described under "MATERIALS AND METHODS". The background for serotonin release which was obtained in the absence of TPA was subtracted from each of the experimental values. Each point represents the mean SE of 5 experiments. Percent inhibition of 40K protein phosphorylation was calculated from the relative intensity of the phosphorylated protein band of autoradiogram. Each point represents the mean of 3 experiments.
●—● serotonin secretion,
■—■ 40K protein phosphrylation.

From the kinetic analysis, K252a inhibits protein kinase C by competing with ATP but not with phosphatidylserine (13). In our experiments, H-7 (10-100 μM) and trifluoperazine (3-30 μM) also inhibited the TPA-induced ATP secretion (data not shown). Therefore, inhibitory effect on the dense granule secretion may be common in the two classes of inhibitors of protein kinase C. The parallel inhibition of 40K protein phosphorylation and serotonin secretion by K252a strongly supports the idea that the former may play a causative role in the latter in TPA-stimulated platelets as proposed by Sano et al (3).

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