Vol. 190, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1993 Pages 263-269

PROTEIN KINASE C INHIBITS THE Ca^{2+}-ACTIVATED K^+ CHANNEL OF CULTURED PORCINE CORONARY ARTERY SMOOTH MUSCLE CELLS

Kazushi Minami [1], Kenji Fukuzawa [1*] and Yutaka Nakaya [2]

[1] Laboratory of Health Chemistry, Faculty of Pharmaceutical Sciences, Tokushima University, Tokushima 770, Japan

[2] Second Department of Internal Medicine, School of Medicine, Tokushima University, Tokushima 770, Japan

Received November 30, 1992

SUMMARY. The effect of protein kinase C (C-kinase) on the Ca^{2+}-activated K^+ channel (K_{Ca}-channel) was studied in cultured smooth muscle cells from porcine coronary artery by the patch-clamp technique. In cell-attached patches, bath application of phorbol 12-myristate 13-acetate (PMA, 1 μM), a C-kinase activator, significantly decreased the open probability of the activated K_{Ca}-channel in the presence of the calcium ionophore A23187 (20 μM), which increases intracellular Ca^{2+}. This decrease in the open probability was reversed by subsequent application of staurosporine (1 nM), a C-kinase inhibitor. Application of 1-oleoyl-2-acetylglycerol (OAG, 30 μM) or 1,2-dioctanoylglycerol (DG8; 30 μM), activators of C-kinase, also inhibited K_{Ca}-channel activation by A23187, and these inhibitions were also reversed by staurosporine. PMA (1 μM) also inhibited K_{Ca}-channel activation by dibutylyl cyclic AMP (db-cAMP, 2 mM) or caffeine (30 mM). In inside-out patches, bath application of the C-kinase fraction from rat brain in the presence of ATP (1 mM) and PMA (1 μM) markedly inhibited the K_{Ca}-channel. These results indicate that activation of C-kinase inhibits the K_{Ca}-channel and may cause membrane depolarization and vascular contraction.

Activation of protein kinase C (C-kinase) causes contraction of vascular smooth muscle (1-5). This contraction is supposed to be partially dependent on Ca^{2+} influx from the extracellular fluid,

* To whom correspondence should be addressed.

0006-291X/93 $4.00

because C-kinase activators increase the intracellular Ca^{2+} concentration (2,4,5), but the mechanism of Ca^{2+} influx induced by C-kinase is not clear.

C-Kinase is supposed to affect ion channels in the cell membrane and recently it was shown to inhibit the ATP-sensitive K^+ channel (K_{ATP}-channel) in cells of the rat cortical collecting duct (6). The membrane potential is mainly controlled by K^+ channels. The opening of K^+ channels causes membrane hyperpolarization and smooth muscle relaxation (7). In vascular smooth muscle cells, the large conductance Ca^{2+}-activated K^+ channel (K_{Ca}-channel) and the K_{ATP}-channel are particularly important in control of vascular tone (8). The K_{Ca}-channel is activated by depolarization and increase in intracellular Ca^{2+}, and counteracts contraction of smooth muscle. The K_{Ca}-channel in vascular smooth muscle is also reported to be modulated by cAMP-dependent protein kinase (9,10). However, modulation of the K_{Ca}-channel by C-kinase has not yet been shown. In this paper, we report the inhibitory effect of C-kinase on the K_{Ca}-channel in porcine coronary artery smooth muscle cells.

MATERIALS AND METHODS

Cell culture. Smooth muscle cells were obtained from porcine coronary artery as described (11). Coronary arteries were excised from fresh porcine heart, and cut into small pieces after removing the endothelial tissue. These pieces were then placed on glass coverslips in tissue culture dishes filled with medium [Medium 199 (Nissui Chemicals, Hiroshima, Japan) supplemented with 10 % fetal bovine serum (GIBCO, Grand Island, NY), 100 μg/ml of streptomycin and 100 μg/ml of penicillin]. The smooth muscle cells that migrated out of the tissue blocks were used for experiments after their culture for 6 to 10 days. No proteolytic enzyme was used for isolation of single cells.

Electrical measurements. Experiments were conducted on cell-attached membrane patches as described by Hamill et al. (12). Single channel currents were recorded with a patch clamp amplifier (Nihon Kohden). Currents were low-pass filtered at 3 kHz. Heat-polished glass patch electrodes with a tip resistance of 5 to 7 megaohms were used. The seal resistance between the pipette tip and the cell membrane was 10 to 20 gigaohms. The patch pipette solution contained (in mM): NaCl 140, KCl 2.7, $MgCl_2$ 1, Na-[3-{N-morpholino}-propanesulfonic acid (MOPS)] 10, glucose 5, and free Ca^{2+} 0.001. The bath solution contained (in mM):

K-aspartate 100, KCl 40, K-MOPS 10, and an appropriate amount of free Ca^{2+}. All experiments were performed at 34-37°C.

Data analysis. Open probability (Po) measured from current amplitude histogram was calculated from the following equation

$$Po = {}^{1}/_{N} \sum_{n=0}^{N} (n \cdot Pn)$$

where N is the number of channels in the patch. Results are expressed as means ± S.E.M.s.

Purification of protein kinase C. C-Kinase from rat brain was partially purified as described by Kitano et al. (13). Briefly, a homogenate of brain was centrifuged at 100,000 x g at 4 °C. The supernatant was then subjected to DEAE-cellulose ion exchanger liquid chromatography, and the fraction containing C-kinase was eluted with Tris-HCl buffer (pH 7.5) containing 90 mM NaCl. The activity of C-kinase was determined with an enzyme assay kit (Amersham, Tokyo, Japan).

Drugs. PMA, OAG and calcium ionophore A23187 were obtained from Sigma (St. Louis, MO). Staurosporine and DG8 were from Funakoshi (Tokyo, Japan). PMA, OAG, and DG8 were dissolved in ethanol, and staurosporine and A23187 in dimethylsulfoxide for addition to the bath. The final concentrations of organic solvents were less than 1 %, which had no effect on the opening of the K_{Ca}-channel.

RESULTS

In cell-attached patches of smooth muscle cells from porcine coronary artery, the K_{Ca}-channel had a conductance of 150 pS between -20 and +40 mV with 2.7 mM K^+ on the pipette solution. We tested the effects of C-kinase activators on the K_{Ca}-channel activated by several activators such as calcium ionophore A23187, caffeine and dibutylyl cAMP (db-cAMP), because the open probability of this channel was very low in the control state (less than 0.001).

Figure 1 shows the effects of the C-kinase activators PMA, OAG and DG8 on the K_{Ca}-channel in the presence of A23187 in the bath at a patch membrane potential of +20 mV from the resting potential (pipette potential = -20 mV). Application of A23187 (20 μM) to the bathing solution, increased the open probability to 0.242±0.029 (n=10, $P<0.01$). Bath application of PMA (1 μM) significantly inhibited the activity of the K_{Ca}-channel activated

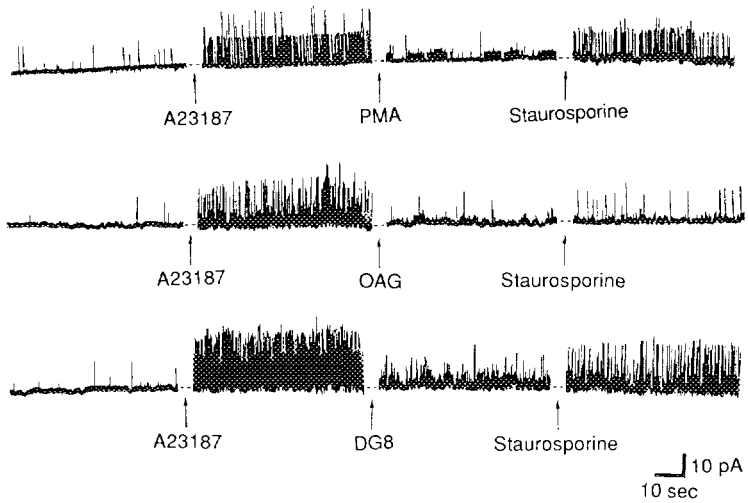

Fig. 1. Inhibitory effects of PMA, OAG and DG8 on the K_{Ca}-channel activated by A23187 in cell-attached membrane patches and reversal of the inhibitions by staurosporine. The pipette holding potential was -20 mV (the patch membrane potential was +20 mV from the resting potential). A23187 and staurosporine were added to the bath at 20 μM and 1 nM, respectively. The solution in the pipette contained 140 mM NaCl,2.7 mM KCl, 1 mM $MgCl_2$, 10 mM K-MOPS, 5 mM glucose, and 1 μM of free Ca^{2+}. The bath solution contained 100 mM K-aspartate, 40 mM KCl, 10 mM K-MOPS and 2 mM $CaCl_2$. The concentrations of PMA, OAG and DG8 added to the bath were 1 μM, 30 μM and 30 μM, respectively.

by A23187, decreasing the open probability to less than 0.001 (n=4, $P<0.05$). This inhibition was reversed by subsequent application of staurosporine (1 nM), with increase in the open probability to 0.168±0.055 ($P<0.05$). The transmembrane permeable DGs had similar effects to PMA, the open probabilities of the K_{Ca}-channel treated with OAG (n=3, $P<0.05$) and DG8 (n=3, $P<0.05$) being less than 0.001. These DG-induced decreases of K_{Ca}-channel activity were also reversed by 1 nM staurosporine to open probabilities of 0.118±0.052 (OAG) and 0.115±0.056 (DG8). These results indicate that C-kinase inhibits the K_{Ca}-channel after its activation by an increased concentration of intracellular free Ca^{2+}.

In rat aortic smooth muscle cells, db-cAMP is reported to activate A-kinase resulting in activation of the K_{Ca}-channel (9). The K_{Ca}-channel was also activated by db-cAMP (2 mM) in our preparation and the activation was inhibited by PMA (1 μM), which decreased the open probability from 0.197 to less than

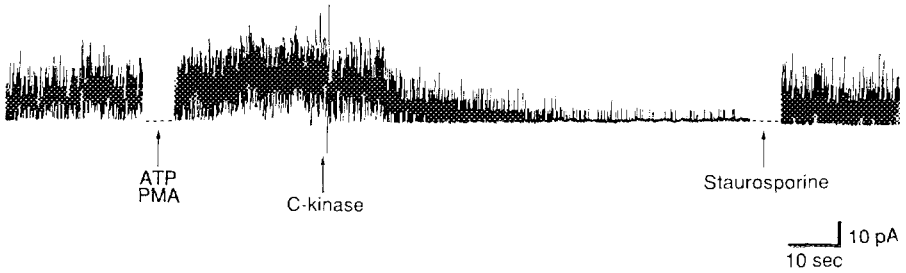

Fig. 2. Inhibitory effect of C-kinase on the K_{Ca}-channel in the presence of ATP and PMA in inside-out membrane patches and reversal of the inhibition by staurosporine. The holding potential was 0 mV. The pipette solution was as for Fig. 1. The bath solution was also as for Fig. 1 but with 1 μM free Ca^{2+}. ATP, PMA, and C-kinase fractions were added to the bath at 1 mM, 1 μM and 1 units/ml, respectively.

0.001. PMA also inhibited the K_{Ca}-channel activated by caffeine, which increases the intracellular Ca^{2+} level by causing Ca^{2+} mobilization from intracellular stores (data not shown).

Finally, we investigated the direct effect of C-kinase on the K_{Ca}-channel using excised inside-out patches. The K_{Ca}-channel activated by 10^{-6} M Ca^{2+} was inhibited by application of the C-kinase fraction (1 units/ml) from rat brain to the bath (cytosolic side) in the presence of 1 mM ATP and 1 μM PMA at a membrane potential of 0 mV (Fig. 2); the open probability of the K_{Ca}-channel was decreased from 0.339±0.015 to 0.005±0.002 (n=4, $P<0.001$). The C-kinase fraction alone did not inhibit the K_{Ca}-channel in the absence of ATP and PMA (data not shown), which are required to activate phosphorylation by C-kinase. The inhibition of the K_{Ca}-channel by C-kinase was reversed by subsequent application of staurosporine (1 nM), with increase in the open probability to 0.236±0.149.

DISCUSSION

Activation of C-kinase causes increase in the intracellular Ca^{2+} concentration and smooth muscle contraction (1-5). Recently, the ATP-sensitive K^+ channel was reported to be inhibited by C-kinase (6). However, it has not been shown whether C-kinase modulates the K_{Ca}-channel, which counteracts the vasoconstriction and is activated by increase of intracellular Ca^{2+} and depolarization. This study is the first report showing that C-kinase inhibits the K_{Ca}-channel.

We found that in cell-attached patches, C-kinase activators (PMA, OAG and DG8) inhibited the K_{Ca}-channel activated by increase in the cytosolic free Ca^{2+} by A23187 (Fig. 1). We also confirmed the inhibition of the K_{Ca}-channel by activated C-kinase in inside-out patches using a C-kinase fraction from rat brain in the presence of ATP and PMA (Fig. 2). These inhibitory effects were reversed by staurosporine. These results indicate that activated C-kinase inhibits the K_{Ca}-channel directly, probably via phosphorylation of the channel protein. The K_{Ca}-channel activated by db-cAMP or caffeine was also inhibited by PMA. Thus C-kinase inhibits the K_{Ca}-channel activated by either Ca^{2+} or A-kinase.

The present results indicate that activation of C-kinase inhibits the K_{Ca}-channel, and may cause concurrent Ca^{2+} influx through the voltage-dependent Ca^{2+} channel by membrane depolarization, because inhibitors of the K_{Ca}-channel are reported to contract smooth muscles of aorta and vein (14,15). This inhibition of the K_{Ca}-channel may be involved in the mechanism of C-kinase-induced vasoconstriction. We are now studying the inhibition of K_{Ca}-channel and associated activation of C-kinase by vascular contractile agonists such as endothelin and angiotensin II.

Here we report inhibition of the K_{Ca}-channel by C-kinase. Recently, we demonstrated activation of this channel by A-kinase (10). These observations indicate that the K_{Ca}-channel is phosphorylated at different sites by A- and C-kinase, and so may have a dual action in regulating contraction of vascular smooth muscle.

ACKNOWLEDGMENTS

We wish to thank Dr. I. Inoue (Institute for Enzyme Research, Tokushima University) for constructive advice, Dr. Y. Yoshimura (Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tokushima University) for purification of C-kinase, and Mr. Y. Hirata (Laboratory of Health Chemistry, Faculty of Pharmaceutical Sciences, Tokushima University) for technical assistance in this study.

REFERENCES

1. Baraban, J.M., Gould, R.J., Peroutka, S.J. and Snyder, S.H. (1985) Proc. Natl. Acad. Sci. USA 82, 604-607.
2. Singer, H.A. and Baker, K.M. (1987) J. Pharmacol. Exp. Ther. 243, 814-821.

3. Khalil, R.A. and Van Breemen, C. (1987) J. Pharmacol. Exp. Ther. 244, 537-542.
4. Chiu, P.J.S., Tetzloff, G., Chatterjee, M. and Sybertz, E.J. (1988) Naunyn-Schmiedeberg's Arch. Pharmacol. 338, 114-120.
5. Mori, T., Yanagisawa, T. and Taira, N. (1990) Naunyn-Schmiedeberg's Arch. Pharmacol. 341, 251-255.
6. Wang, W. and Giebisch, G. (1991) Proc. Natl. Acad. Sci. USA 88, 9722-9725.
7. Westen, A.H. (1989) Pflügers Arch. 414 (Suppl 1), S99-S105.
8. Wakatsuki, T., Nakaya, Y. and Inoue, I. (1992) 263, H618-H623.
9. Sadoshima, J., Akaike, N., Kanaide, H. and Nakamura, M. (1988) Am. J. Physiol. 255, H754-H759.
10. Minami, K., Fukuzawa, K., Nakaya, Y., Zeng, X.R. and Inoue, I. (submitted).
11. Inoue, I., Nakaya, Y., Nakaya, S. and Mori, H. (1989) FEBS Lett. 255, 281-284.
12. Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers Arch. 391, 85-100.
13. Kitano, T., Go, M., Kikkawa, U. and Nishizuka, Y. (1986) Meth. Enzymol. 124, 349-352.
14. Hu, S., Kim, H.S., Okolie, P. and Weiss, G.B. (1990) J. Pharmacol. Exp. Ther. 253, 771-777.
15. Suarez Kurtz, G., Garcia, M.L. and Kaczorowski, G.J. (1991) J. Pharmacol. Exp. Ther. 259, 439-443.