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PROTEIN KINASE C-INDEPENDENT INHIBITION OF THE Ca^{2+}-ACTIVATED K^+ CHANNEL BY ANGIOTENSIN II AND ENDOTHELIN-1

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Abstract—We previously reported that the Ca^{2+}-activated K^+ channel (K_{Ca}-channel) in cultured smooth muscle cells from porcine coronary artery was inhibited by protein kinase C (C-kinase). In this study, inhibition of the K_{Ca}-channel by receptor-mediated vascular contractile agonists, such as angiotensin II (ANG II) and endothelin-1 (ET-1), was investigated by the patch-clamp technique. In cell-attached patches, addition of ANG II (500 nM) or ET-1 (50 nM) to the bath inhibited the K_{Ca}-channel activated by the calcium ionophore A23187 (10–20 μM). Phorbol 12-myristate 13-acetate (PMA, 1 μM), a C-kinase activator, also decreased the open probability of the K_{Ca}-channel. The PMA-induced decrease in the open probability was reversed by subsequent application of staurosporine (1 nM), a C-kinase inhibitor, but the ANG II- and ET-1-induced decreases were not reversed by subsequent application of staurosporine (>30 nM). Pretreatment of smooth muscle cells with 30 nM staurosporine, a protein kinase inhibitor, or 1 mM neomycin, an inhibitor of phospholipase C, also did not abolish the inhibition of the K_{Ca}-channel by ANG II. Furthermore, ANG II inhibited the K_{Ca}-channel in cells in which C-kinase was down-regulated. These results indicate that, in porcine coronary artery smooth muscle cells, ANG II and ET-1 inhibit the K_{Ca}-channel by a C-kinase-independent mechanism.

Key words: Ca^{2+}-activated K^+ channel; angiotensin II; endothelin-1; protein kinase C; porcine coronary artery smooth muscle cells

The Ca^{2+}-activated K^+ channel (K_{Ca}-channel§) shows large unitary conductance [1, 2] and plays an important role in the relaxation of vascular smooth muscle and the control of vascular tone. Consequently, inhibition of this channel induces tonic vascular contraction [3, 4]. We found previously that the K_{Ca}-channel of porcine coronary artery smooth muscle cells is inhibited by activation of C-kinase [5]. In our previous study, C-kinase activators such as PMA and two DGs, OAG and DG8, were shown to inhibit the K_{Ca}-channel.

ANG II and ET-1 are potent vasoactive substances known to activate C-kinase via generation of DG by hydrolysis of phosphatidylinositol 4,5-bisphosphate [6]. These substances increase the intracellular Ca^{2+} concentration, and induce activation of C-kinase and several other responses for vascular contraction [6]. The increase of the intracellular Ca^{2+} concentration induces vasoconstriction, but activates K_{Ca}-channels. Thus, agonist-induced contraction may be enhanced by the inhibition of K_{Ca}-channels. Therefore, we expected that ANG II and ET-1 would inhibit the K_{Ca}-channel in vascular smooth muscle cells.

In this study, we investigated the inhibitory effects of the receptor-mediated vascular contractile agonists ANG II and ET-1 on the K_{Ca}-channel of porcine coronary artery smooth muscle cells, and found C-kinase-independent K_{Ca}-channel inhibition.

MATERIALS AND METHODS

Materials. ANG II, PMA, OAG, calcium ionophore A23187, neomycin, IP_3, IP_4, 1,2-dilauroylglycerol and 1,2-dilauroylphosphatidic acid were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Staurosporine was from Funakoshi (Tokyo, Japan). ET-1 was purchased from the Peptide Institute Inc. (Osaka, Japan). PMA was dissolved in ethanol, and staurosporine and A23187 were dissolved in dimethyl sulfoxide for addition to the bath. The final concentrations of organic solvents were less than 1.2%, which had no effect on the opening of the K_{Ca}-channel.

Cell culture. Smooth muscle cells were obtained from porcine coronary arteries, as described [7]. Briefly, coronary arteries were excised from fresh porcine hearts, 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

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§ Abbreviations: K_{Ca}-channel, Ca^{2+}-activated K^+channel; C-kinase, protein kinase C; ANG II, angiotensin II; ET-1, endothelin-1; PMA, phorbol 12-myristate 13-acetate; DG, diacylglycerol; OAG, 1-oleoyl-2-acetylglycerol; DG8, 1,2-dioctanoylglycerol; IP_3, inositol 1,4,5-trisphosphate; IP_4, inositol 1,3,4,5-tetrakisphosphate; PA, phosphatidic acid; and NPo, open probability.

10% fetal bovine serum (GIBCO, Grand Island, NY, U.S.A.), 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 culturing for 6–10 days. No proteolytic enzyme was used for the isolation of single cells.

Electrical measurements. Experiments were conducted on cell-attached or inside-out membrane patches as described by Hamill *et al.* [8]. Single channel currents were recorded with a patch clamp amplifier (Nihon Kohden). Currents were low-pass filtered at 3 kHz. Glass patch electrodes with a tip resistance of 5–7 megaohms were used. The seal resistance between the pipette tip and the cell membrane was 10–20 gigaohms. The patch pipette solution contained 140 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 10 mM sodium-[3-{*N*-morpholino}-propanesulfonate] (MOPS), 5 mM glucose, and 1 μM free Ca^{2+} (1 mM EGTA and 920 μM $CaCl_2$). The bath solution contained 100 mM potassium-aspartate, 40 mM KCl, 10 mM K-MOPS and 2 mM $CaCl_2$ (for cell-attached patches) or 1 μM free Ca^{2+} (for inside-out patches). All solutions were adjusted to pH 7.2 with NaOH or KOH, and experiments were performed at 34–37°.

Data analysis. NPo measured from current amplitude histograms was calculated from the following equation:

$$\mathrm{NPo} = \sum_{n=0}^{N} (n \cdot \mathrm{Pn})$$

where n is the number of channels in the open state, N is the maximum number of open state channels in a patch, and Pn is the area of the peak in the histogram. Observed NPo values are expressed as percentages of that of the K_{Ca}-channel activated by 10–20 μM A23187. The mean NPo of the A23187-activated K_{Ca}-channel was 0.833 ± 0.173 (N = 13). All values are means ± SEM. Student's *t*-test for paired data was used for statistical analysis, and values of $P < 0.05$ were considered significant.

RESULTS

In cell-attached patch configurations of smooth muscle cells from porcine coronary arteries, ANG II and ET-1 were added to the bath in the presence of the calcium ionophore A23187 because of the low activity of K_{Ca}-channels in the patch membrane (open probability < 0.01).

As reported previously [5], PMA inhibited the K_{Ca}-channel in the presence of A23187, and this inhibition was reversed by subsequent application of 1 nM staurosporine (Fig. 1A). Similar results were obtained with the transmembrane permeable DGs, OAG and DG8 (data not shown). These results indicate that C-kinase activators inhibit the K_{Ca}-channel activated by an increased concentration of intracellular free Ca^{2+}. ANG II, which activates C-kinase [6], markedly decreased the K_{Ca}-channel activity in the presence of 10–20 μM A23187 (Fig. 1B). However, this decrease by ANG II was not reversed by staurosporine, even upon its addition in 30-fold excess (Fig. 1B). Staurosporine (1 μM) did not inhibit significantly the decrease of K_{Ca}-channel activity induced by ANG II (data not shown). ET-1 also inhibited the K_{Ca}-channel, and its inhibition, likewise, was not reversed by 30 nM staurosporine (N = 3). ANG II inhibited the K_{Ca}-channel in smooth muscle cells 5 min after the addition of 30 nM staurosporine. Other protein kinase inhibitors (100 μM H-7, another C-kinase inhibitor, and 10 μg/mL genistein, a specific inhibitor of tyrosine kinase) did not reverse the inhibition by ANG II (data not shown).

Figure 2 shows the inhibitory effects of PMA, ANG II and ET-1 on the K_{Ca}-channel activated by A23187 and the effects of staurosporine on this inhibition. In this figure, the mean NPo of the A23187-activated K_{Ca}-channel (0.833 ± 0.173; N = 13) is taken as 100%. The percent NPo was reduced to 0.39 ± 0.20% by 1 μM PMA, 1.96 ± 0.82% by 500 nM ANG II, and 30.27 ± 13.36% by 50 nM ET-1. Upon addition of 30 nM staurosporine, the decrease in channel activity by ANG II or ET-1 was not reversed (0.93 ± 0.60 and 36.94 ± 10.63%, respectively), whereas the decrease by PMA was reversed appreciably by 1 nM staurosporine (66.20 ± 16.60%, $P < 0.05$).

ANG II and ET-1 are known to activate phospholipase C and to produce two second messengers, IP_3 and DG, by hydrolysis of phosphatidylinositol 4,5-bisphosphate [6, 9]. IP_3 is then metabolized to IP_4 by IP_3 3-kinase [10, 11], and DG to PA by DG-kinase [12]. We therefore tested the effects of these second messengers, IP_3 and DG, and their metabolites, IP_4 and PA, on the K_{Ca}-channel activated by 1 μM Ca^{2+} by the inside-out patch-clamp technique. Results showed no inhibition of the K_{Ca}-channel by these second messengers or their metabolites at a concentration of 10 μM (Fig. 3).

Figure 4 shows the effect of ANG II on the K_{Ca}-channel in smooth muscle cells with down-regulated C-kinase, prepared by preincubation with 500 nM PMA for 18–24 hr. The A23187-activated K_{Ca}-channel of these cells was inhibited by bath application of ANG II. On the contrary, OAG, which is reported to inhibit the K_{Ca}-channel of normal cells via activation of C-kinase [5], did not inhibit the K_{Ca}-channel in these cells.

Figure 5 shows the inhibitory effects of ANG II on the K_{Ca}-channel in smooth muscle cells with down-regulated C-kinase or pretreated with 30 nM staurosporine or 1 mM neomycin, an inhibitor of phospholipase C. The percent NPo of the K_{Ca}-channel was inhibited significantly, but not completely, by 1 μM ANG II; the remaining percentages were 15.2 ± 7.1 (with 500 nM PMA), 15.5 ± 6.5 (with 30 nM staurosporine) and 22.6 ± 6.7 (with 1 mM neomycin).

DISCUSSION

Hu *et al.* [13] reported that ET-1 inhibits the K_{Ca}-channel of porcine coronary artery smooth muscle cells, and Kanazirska *et al.* [14] observed that ANG II inhibits the inward rectifier K^+ channel of rat and bovine adrenal glomerulosa cells. Both groups speculated that C-kinase participates in inhibition of these channels. On the contrary, Benz *et al.* [15]

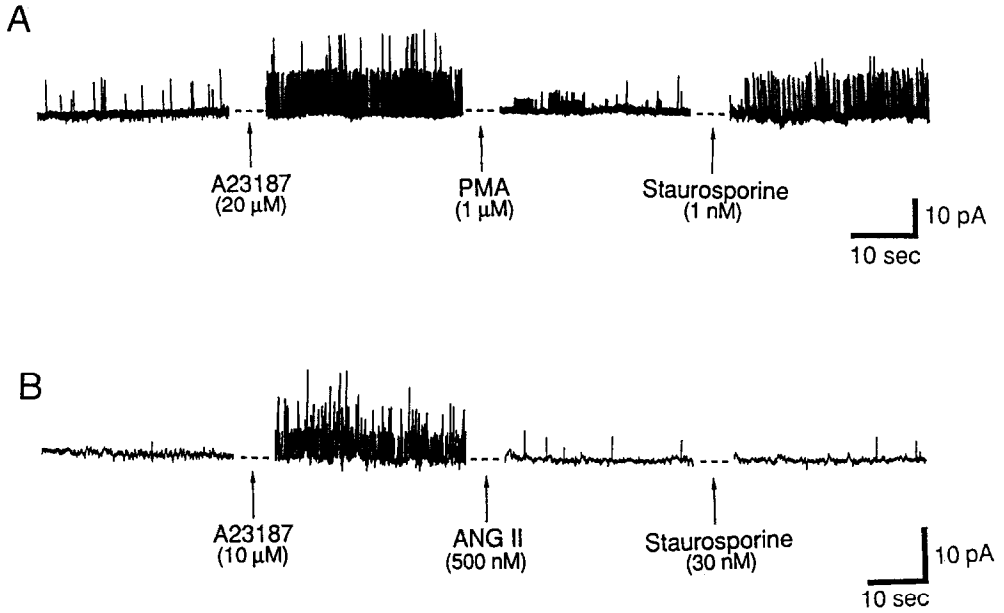

Fig. 1. Effects of staurosporine on inhibition of the A23187-activated K_{Ca}-channel by PMA (A) and ANG II (B) in cell-attached membrane patches. The solution in the pipette contained 140 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 10 mM K-MOPS, 5 mM glucose, and 1 μM free Ca^{2+}. The bath solution contained 100 mM potassium-aspartate, 40 mM KCl, 10 mM K-MOPS and 2 mM $CaCl_2$. Results are representative of four to five preparations. (**A.**) The pipette holding potential was −20 mV (the patch membrane potential was +20 mV from the resting potential). A23187, PMA and staurosporine were added to the bath at 20 μM, 1 μM and 1 nM, respectively. (**B.**) The pipette holding potential was 0 mV. A23187, ANG II and staurosporine were added to the bath at 20 μM, 500 nM and 30 nM, respectively.

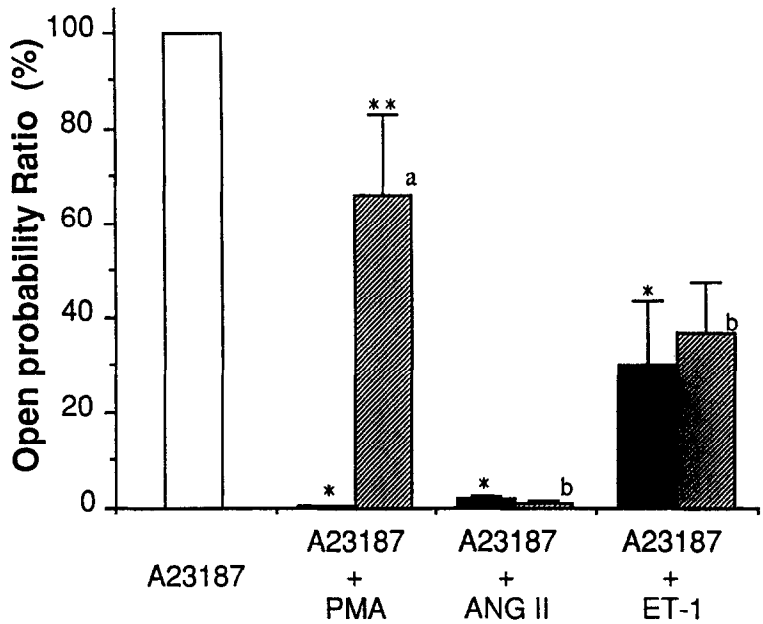

Fig.2. Inhibitory effects of PMA, ANG II and ET-1 on the K_{Ca}-channel activated by A23187 in cell-attached membrane patches and the effects of staurosporine on this inhibition. The pipette holding potential was −20 mV. The pipette and bath solutions were as described for Fig. 1. Values are shown as percentages of the NPo of the K_{Ca}-channel activated by 10–20 μM A23187 (□). PMA, ANG II and ET-1 were added to the bath at 1 μM, 500 nM and 50 nM, respectively (■). Staurosporine (▨) was subsequently added to the bath at 1 nM [in the presence of PMA (a)] or 30 nM [in the presence of ANG II or ET-1 (b)]. Values are means ± SEM for three to five experiments. Key: (*) $P < 0.05$, vs the NPo of the K_{Ca}-channel activated by A23187; and (**) $P < 0.05$, vs the NPo in the presence of PMA but not staurosporine.

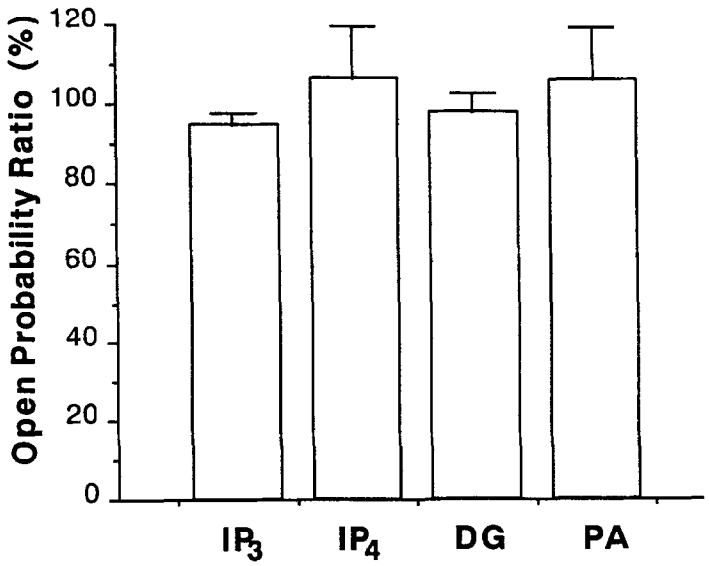

Fig. 3. Absence of inhibitory effects of IP_3, IP_4, DG and PA on K_{Ca}-channels in inside-out patches. The bath solution contained 100 mM potassium-aspartate, 40 mM KCl, 10 mM K-MOPS and 1 μM free Ca^{2+} (prepared with 920 μM $CaCl_2$ and 1 mM EGTA). The pipette solution was as described for Fig. 1. Pipette holding potentials were all −20 mV. All second messengers were added to the bath at 10 μM. Values are shown as percentages of the NPo of the K_{Ca}-channel activated by 1 μM free Ca^{2+}. The NPo of control patches was 1.68 ± 0.35 (N = 12). Values are means ± SEM for three experiments.

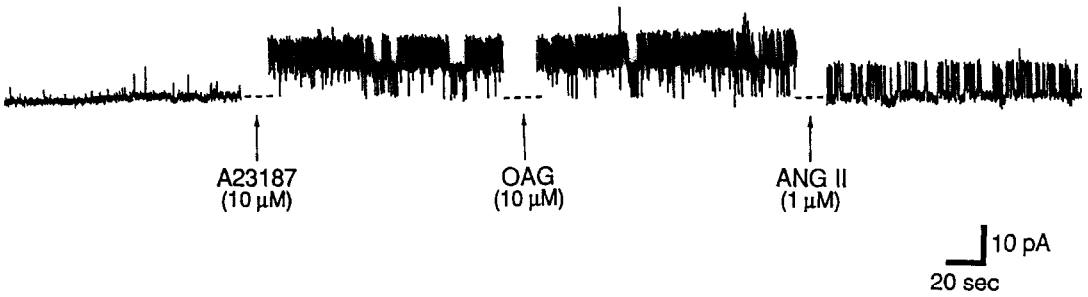

Fig. 4. Inhibitory effect of ANG II on the K_{Ca}-channel of smooth muscle cells with down-regulated C-kinase. Cultured porcine coronary artery smooth muscle cells were pretreated with 500 nM PMA for 18–24 hr in the conditions for cell culture, and washed before patch-clamp experiments. Experiments were performed on cell-attached membrane patches. The pipette holding potential was −20 mV. The pipette and bath solutions were as described for Fig. 1. A23187, OAG and ANG II were added to the bath at 10, 10 and 1 μM, respectively.

reported that ANG II activates the cardiac Na^+ channel by some unknown C-kinase-independent mechanism. In this study, we tested whether receptor-mediated vascular contractile agonists inhibited the K_{Ca}-channel via C-kinase activation.

We found that the K_{Ca}-channel was inhibited by ANG II or ET-1 as well as by PMA. However, staurosporine did not reverse the inhibition by ANG II or ET-1, whereas it significantly inhibited that by PMA (Figs. 1 and 2). Moreover, ANG II inhibited the K_{Ca}-channel in smooth muscle cells in which C-kinase was down-regulated (Figs. 4 and 5) or in cells pretreated with staurosporine, a protein kinase inhibitor (Fig. 5). These results indicate that ANG II and ET-1 inhibit the K_{Ca}-channel of porcine coronary artery smooth muscle cells via a C-kinase-independent mechanism. We confirmed that ANG II is unlikely to inhibit the K_{Ca}-channel for lowering of cytosolic Ca^{2+} concentration by showing that application of ANG II and/or staurosporine did not alter the cytosolic free-Ca^{2+} concentration, measured with Fura-2, of porcine coronary artery smooth muscle cells treated with A23187 (data not shown).

We examined this C-kinase-independent mechanism of K_{Ca}-channel inhibition by ANG II and ET-1. Most receptor-mediated vascular contractile

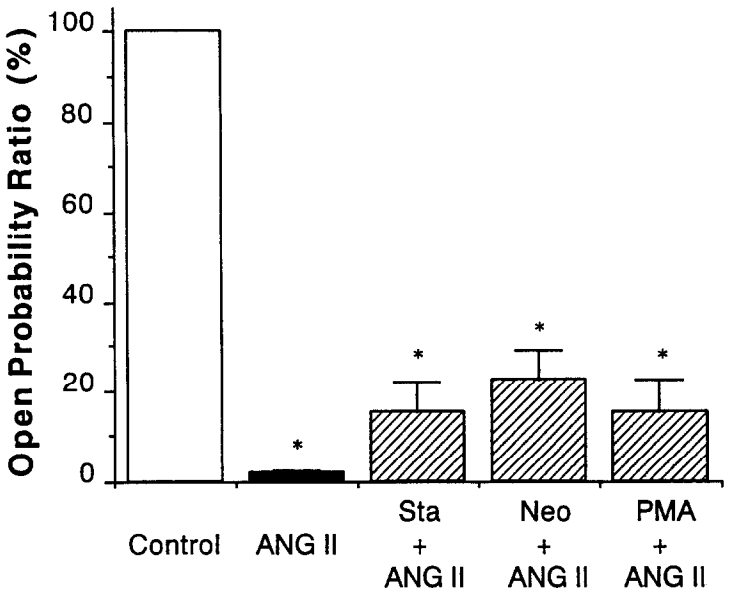

Fig. 5. Inhibitory effect of ANG II on the K_{Ca}-channel of porcine coronary artery smooth muscle cells pretreated with 30 nM staurosporine, 1 mM neomycin or 500 nM PMA. Staurosporine and neomycin were added to the bath 5 and 30 min before the application of ANG II. Pretreatment with PMA to down-regulate C-kinase was carried out as for Fig. 3. The pipette holding potential was −20 mV. The pipette and bath solutions were as described for Fig. 1. Values are shown as percentages of the NPo of the K_{Ca}-channel activated by 10–20 μM A23187 (□). ANG II was added to the bath at 1 μM with (▨) or without (■) C-kinase inactivation. These pretreatments for inactivation of C-kinase did not affect activation of the K_{Ca}-channel by A23187. Values are means ± SEM for four to seven experiments. Key: (*) $P < 0.05$, vs the NPo of the K_{Ca}-channel activated by A23187.

agonists activate phospholipase C, and produce two second messengers, IP_3 and DG [6, 9]. However, IP_3 and DG did not inhibit the K_{Ca}-channel in inside-out membrane patches (Fig. 3). Furthermore, IP_4 and PA, metabolites of IP_3 and DG [10–12], also did not affect the K_{Ca}-channel activities (Fig. 3). In addition, pretreatment with 1 mM neomycin, an inhibitor of phospholipase C [16], failed to prevent the inhibition of the K_{Ca}-channel by ANG II in attached cells (Fig. 5). These results suggest that inhibition of the K_{Ca}-channel by ANG II is not dependent on activation of phospholipase C.

ANG II and ET-1 are known to activate phospholipase D [17–19]. Activation of phospholipase D results in the production of PA and, subsequently, metabolites such as DG [17, 18]. However, in inside-out patches, PA and DG did not inhibit the K_{Ca}-channel. Therefore, activation of phospholipase D is also unlikely to be involved in inhibition of the K_{Ca}-channel by ANG II or ET-1.

ANG II and ET-1 have also been reported to activate tyrosine kinase [19, 20]. However, 10 μg/mL genistein, a tyrosine kinase inhibitor, did not reverse the channel inhibition by ANG II (data not shown). Thus, tyrosine kinase may not participate in K_{Ca}-channel inhibition by ANG II.

In addition, ANG II and ET-1 have been shown to cause direct inhibition of several K^+ channels [21–23]. But it is unlikely that this effect was involved in inhibition of the K_{Ca}-channel by ANG II and ET-1 in this study, because the inhibition was observed in the cell-attached configuration, and agents were added to the bath (with no contact between them and the recording channel). Thus, the C-kinase-independent K_{Ca}-channel inhibition reported here may by due to a novel intracellular signal transduction pathway.

We recently reported that an increase of intracellular cyclic AMP activates the K_{Ca}-channel of porcine coronary artery smooth muscle cells [24], and the activation of C-kinase inhibits this channel [5], and suggested that the K_{Ca}-channel has an important role in the vascular actions of A- and C-kinases. Here we report a novel mechanism of C-kinase-independent K_{Ca}-channel inhibition by ANG II or ET-1 that is also an important mechanism of vascular contraction.

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