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European Journal of Pharmacology 416 (2001) 179–183

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Nicorandil-induced ATP release in endothelial cells of rat caudal artery is associated with increase in intracellular Ca^{2+}

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Received 24 November 2000; received in revised form 14 February 2001; accepted 20 February 2001

Abstract

The effect of nicorandil, an ATP-sensitive K^+ channel opener, on the level of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and on ATP release in endothelial cells of the rat caudal artery was examined using a fluorescent confocal microscopic imaging system and high-performance liquid chromatography (HPLC) with fluorescent detection, respectively. Nicorandil significantly increased $[Ca^{2+}]_i$ and the overflow of ATP and its metabolites. The former reaction was abolished in the absence of extracellular Ca^{2+}, but it did not change in the presence of thapsigargin or cyclopiazonic acid. The increase in the overflow of ATP and $[Ca^{2+}]_i$ induced by nicorandil was markedly suppressed by glibenclamide, an ATP-sensitive K^+ channel blocker. The increase of $[Ca^{2+}]_i$ induced by nicorandil was significantly and inversely correlated with the level of intracellular ATP in the endothelial cells, suggesting that activation of ATP-sensitive K^+ channels by nicorandil increases Ca^{2+} influx in endothelial cells. The increase of $[Ca^{2+}]_i$ might be associated with ATP release.

Keywords: Nicorandil; ATP; Ca^{2+}, intracellular; Endothelial cell; Caudal artery, rat

1. Introduction

Nicorandil has been proposed to have at least two activities: it increases guanosine 3′:5′-cyclic monophosphate levels by stimulating soluble guanylate cyclase in a manner similar to nitroglycerin esters (Endoh and Taira, 1983), and it increases membrane K^+ conductance, leading to cellular hyperpolarization and consequently to closure of voltage-operated Ca^{2+} channels (Furukawa et al., 1981). These events result in vascular smooth muscle relaxation, presumably by an attenuation of intracellular Ca^{2+}. Nicorandil enhances the release of ATP from endothelial cells isolated from the porcine coronary artery (Hashimoto et al., 1999), although the mechanism remains unknown. A large amount of ATP is released from the vascular bed, especially from vascular endothelial cells (Sedaa et al., 1990; Shinozuka et al., 1994), and extracellular ATP induces endothelium-dependent vasodilatation of arteries by binding to P_{2y} or P_{2u} purinoceptors, or both (Dalziel and Westfall, 1994). The released ATP is rapidly degraded to ADP, AMP and adenosine by ectonucleotidases (Shinozuka et al., 1994). In addition to various physiological and pharmacological effects on the cardiovascular system (Ralevic and Burnstock, 1998), adenosine has the potential for being cardioprotective during preconditioning, ischemia and reperfusion (Hori and Kitakaze, 1991). Also, nicorandil possesses cardioprotective properties after ischemia/reperfusion injury through decreased adenosine levels in myocytes and decreased coronary blood flow (Kitakaze et al., 1996). Thus, it is of interest to examine the mechanism of the nicorandil-induced release of ATP from vascular endothelial cells in order to clarify the cardioprotective mechanism of nicorandil. As an initial approach to a better understanding of the pharmacological properties of nicorandil, we examined its effect on intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in endothelial cells isolated from the rat caudal artery.

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)00867-6

2. Material and methods

2.1. Reagents

Nicorandil (2-nicotinamidoethyl-nitrate ester) was kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Cyclopiazonic acid, thapsigargin and luciferin-luciferase were obtained from Sigma (St. Louis, MO, USA). Calcium Green 1/acetoxymethyl ester (Calcium Green 1/AM) was purchased from Molecular Probes (Eugene, OR, USA): each concentration is expressed as the final molar concentration in the physiological saline solution (PSS) bathing the plates.

2.2. Isolation of endothelial cells from rat caudal artery and release experiments

Rats were provided for and killed in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. Male Wistar rats (SLC, Shizuoka, Japan) (6–9 weeks of age) were anaesthetized with sodium pentobarbital (50 mg kg^{-1}) and exsanguinated. Primary cultures of endothelial cells of rat caudal arteries were prepared by the explant method as described (Hashimoto et al., 1995) and used.

To evaluate the release of purines, the cultured endothelial cells ($20–25 \times 10^4$ cells/35-mm dish) in collagen-coated dishes were washed with PSS containing (in mM) 140 NaCl, 4.0 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 10 HEPES and 10 glucose at pH 7.4 adjusted with NaOH. The cells were then incubated in 2 ml of PSS under a continuous stream of 95% O_2/5% CO_2 for 60 min at 37°C. A quantity of 200 μl of the bathing PSS (prestimulation sample) was then collected, the endothelial cells were stimulated with drugs for 5 min and the solution (stimulation sample) was collected. The amount of ATP, ADP, AMP and adenosine in the sample was determined by high-performance liquid chromatography (HPLC) with fluorescence detection as described (Kawamoto et al., 1998).

To measure the intracellular ATP concentration in endothelial cells, immediately after nicorandil increased the $[Ca^{2+}]_i$ to the maximum level, 1 ml of boiled sterile water was added to each cell layer after removal of the PSS from the collagen-coated glass coverslip. The glass coverslip was scraped with a rubber policeman, and the cells were collected completely with additional boiled sterile water; the cells were then centrifuged at 2000 rpm for 20 min. The cellular ATP in the supernatant was measured by the HPLC method described above.

2.3. Measurement of $[Ca^{2+}]i$

Primary cultures of rat arterial endothelial cells on type I collagen-coated glass coverslips (Collagen Research Center, Tokyo, Japan) were loaded with 5 μM Calcium Green 1/AM (Molecular Probes) for 30 min at 37°C. Cells were rinsed three times with PSS and incubated in PSS for an additional 15 min at 37°C in an atmosphere of 95% air/5% CO_2 to complete the hydrolysis of any intact ester linkages on intracellular Calcium Green 1/AM. The loaded coverslip was placed on the stage of an inverted microscope (Nikon ECLIPSE TE 300, Tokyo, Japan), coupled to the Nipkow disk confocal scanner (CSU10, Yokogawa Electric, Tokyo, Japan). An excitation wavelength of 488 nm was provided by an argon–krypton laser (Omnichrome, Chino, CA, USA), and the emitted light was collected with a 510-nm-long pass dichroic reflector and a 515-nm-long pass emission filter through the planfluor objective (×40 or 20, NA = 0.75, 0.5). The fluorescence images were acquired with an ICCD camera (Hamamatsu Photonica, Hamamatsu, Japan). To quantify the Ca^{2+} responses in endothelial cells, images constructed from 195 × 130 pixels were collected every 5 s. The fluorescence intensity was normalized by dividing the peak average fluorescence intensity (F) during the experiment by the average fluorescence intensity at the beginning of each experiment (F_o).

2.4. Real time imaging of ATP release

Primary cultures of rat arterial endothelial cells on type I collagen-coated glass coverslips in PSS at 37°C under an atmosphere of 95% air/5% CO_2 were placed on the stage of an inverted microscope (Nikon ECLIPSE TE 300). The cells were incubated with PSS containing luciferin-luciferase (final concentration: 20 mg/ml) for an additional 15 min at 37°C under an atmosphere of 95% air/5% and then stimulated. The luciferin-luciferase bioluminescence images were acquired with a photon-counting camera (C2400-35, Hamamatsu Photonica) and cumulatively collected every 1 min for a total of 5 min.

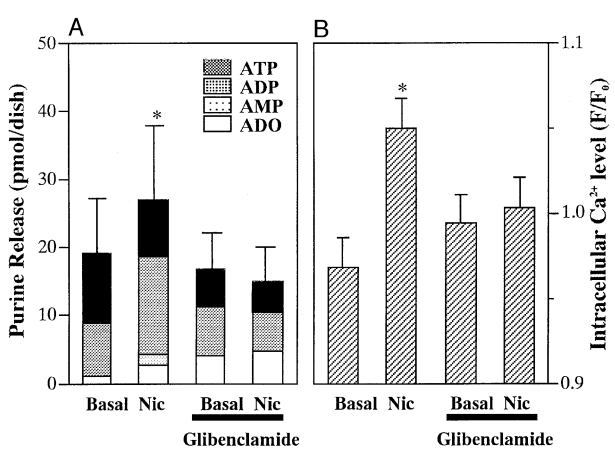

Fig. 1. Effect of nicorandil (Nic) at 100 μM on the release of adenyl purines, ATP, ADP, AMP and ADO (adenosine), (left panel: $n = 4–5$) and on the level of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (right panel: $n = 7–15$) in endothelial cells from rat caudal arteries, and the effect of glibenclamide (100 μM) on both events induced by Nic. Each column shows the mean ± S.E.M. of 10 experiments. $^*P < 0.05$ compared with the basal.

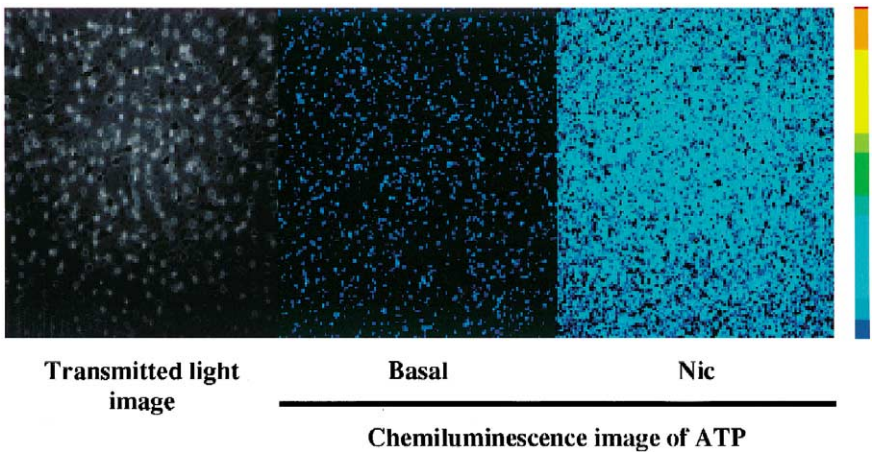

Fig. 2. Chemiluminescence image of ATP release by nicorandil (100 μM) from endothelial cells of rat caudal arteries. Left panel: transmitted light image showing the distribution and morphology of cultured endothelial cells. Phase contrast, ×20 objective. Middle panel: chemiluminescence image of endothelial cells before stimulation with nicorandil showing the basal level of extracellular ATP. Right panel: confocal image of endothelial cells after stimulation with nicorandil showing the increase in extracellular ATP.

2.5. Statistics

Data are expressed as means ± S.E.M. Student's paired *t*-test and analysis of variance (ANOVA) before a post-hoc test of Fisher's Protected Least-Significant Difference (PLSD) was used for comparisons. A probability of less than 0.05 was considered significant. The statistical analyses were carried out with a computer program (Stat View 4.5, Abacus Concepts, California, USA).

3. Results

Stimulation of cultured endothelial cells prepared from rat caudal arteries with 100 μM nicorandil for 5 min significantly increased the release of total adenyl purines (Fig. 1A); however, 10 μM nicorandil did not evoke purine release (data not shown). In the presence of glibenclamide (100 μM), a blocker of the ATP-sensitive K^+ channel, nicorandil did not evoke the release of total adenyl purines; glibenclamide by itself also did not evoke purine release (Fig. 1A). The increase in extracellular ATP induced by nicorandil at 100 μM was also observed with the photon-counting camera (Fig. 2).

Stimulation of the cells with 100 μM nicorandil significantly increased the level of $[Ca^{2+}]_i$ (Fig. 1B), but stimulation with 10 μM nicorandil did not (data not shown). In the presence of glibenclamide (100 μM), nicorandil did not increase the level of $[Ca^{2+}]_i$. Glibenclamide by itself also did not significantly change the level (Fig. 1B).

Stimulation of the endothelial cells with 1 μM ATP significantly increased the level of $[Ca^{2+}]_i$ (Fig. 3). Suramin (100 μM), a P_2 purinoceptor antagonist, significantly reduced the enhancing effect of ATP but did not affect that

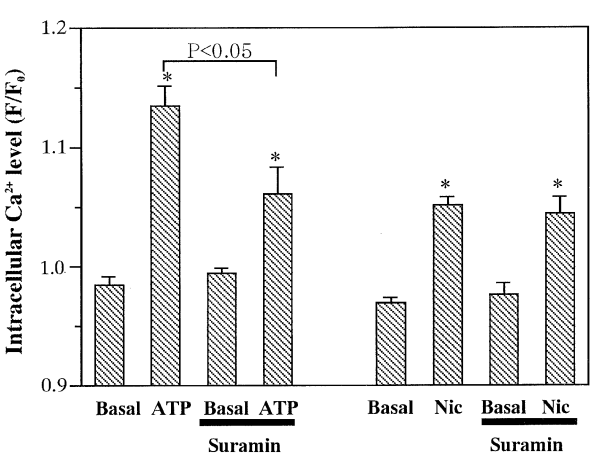

Fig. 3. Effect of suramin (100 μM) on the ATP- and nicorandil (Nic) (100 μM)-induced increase in the intracellular Ca^{2+} level ($[Ca^{2+}]_i$) of endothelial cells from rat caudal arteries. Each column shows the mean ± S.E.M. of 6–15 experiments. $^*P < 0.05$ compared with the basal level.

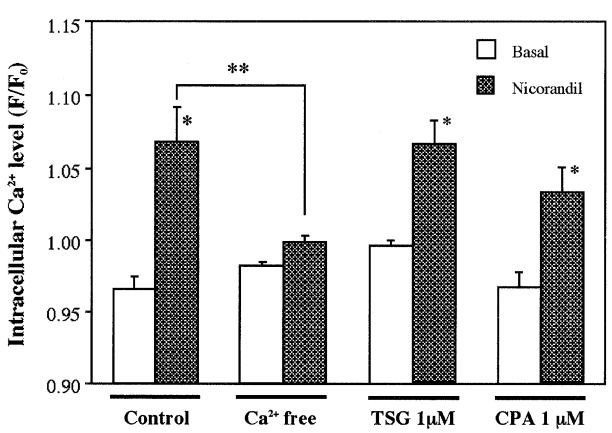

Fig. 4. Effects of Ca^{2+}-free solution, thapsigargin (TSG; 1.0 μM) and cyclopiazonic acid (CPA; 1.0 μM) on the nicorandil (Nic; 100 μM)-induced increase in the intracellular Ca^{2+} level ($[Ca^{2+}]_i$) of endothelial cells from rat caudal arteries. Each column shows the mean ± S.E.M. of 5–15 experiments. $^*P < 0.05$ compared with the basal.

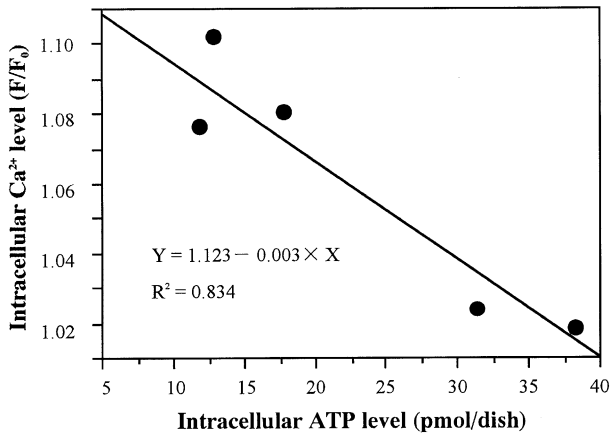

Fig. 5. Relationship between the nicorandil (100 μM)-induced increase in the intracellular Ca^{2+} level ($[Ca^{2+}]_i$) and the intracellular ATP level of endothelial cells from rat caudal arteries.

of nicorandil (100 μM) (Fig. 3). Furthermore, the increase in $[Ca^{2+}]_i$ level was abolished in the absence of extracellular Ca^{2+}, as shown in Fig. 4. Thapsigargin or cyclopiazonic acid (Ca^{2+} pump inhibitors) at 1 μM did not affect the level of $[Ca^{2+}]_i$ (Fig. 4). The increase in $[Ca^{2+}]_i$ induced by ATP was inhibited by thapsigargin and cyclopiazonic acid, but not in the absence of extracellular Ca^{2+} (data not shown).

Regression analysis revealed a significant inverse relationship ($r = -0.914$, $n = 5$) between intracellular ATP levels and the nicorandil-induced $[Ca^{2+}]_i$ level in the endothelial cells (Fig. 5).

4. Discussion

To our knowledge, this is the first study demonstrating that nicorandil, an ATP-sensitive K^+ channel opener, increases $[Ca^{2+}]_i$ in vascular endothelial cells and that this increase is closely associated with the release of ATP from the cells.

The presence of the ATP-sensitive K^+ channel has been demonstrated in endothelial cells from the rat aorta and brain microvessels (Janigro et al., 1993) and in endothelial cells from the rabbit aorta and pulmonary arteries (Katnik and Adams, 1995). K^+ channels are opened as intracellular ATP levels decrease and are blocked by glibenclamide. In the present study, the nicorandil-induced increase of $[Ca^{2+}]_i$ in the endothelial cells of the rat caudal artery was blocked by glibenclamide, and it correlated inversely with the intracellular ATP level. Thus, the nicorandil-induced $[Ca^{2+}]_i$ was highest in endothelial cells where the ATP level was lowest, suggesting that ATP-sensitive K^+ channels participate in Ca^{2+} mobilization in rat caudal arterial endothelial cells.

Indeed, endothelial cell $[Ca^{2+}]_i$ increases after cell hyperpolarization (Lückhoff and Busse, 1990). Furthermore, the activation of endothelial cell ATP-sensitive K^+ channels and subsequent membrane hyperpolarization contribute to the endothelium-dependent regulation of vascular smooth muscle tone (Katnik and Adams, 1997). Changes in $[Ca^{2+}]_i$ are determined by its release from intracellular stores and by its entry through the plasma membrane. Hyperpolarization may act as a driving force for the influx of Ca^{2+}. In the present study, the increase in $[Ca^{2+}]_i$ was abolished in the absence of extracellular Ca^{2+}, but was not affected by the Ca^{2+} pump inhibitors thapsigargin and cyclopiazonic acid. ATP is known to stimulate purinoceptors coupled to phospholipase C and to increase $[Ca^{2+}]_i$ (Dalziel and Westfall, 1994). In cultured endothelial cells from the rat caudal artery, the increase in $[Ca^{2+}]_i$ was inhibited by thapsigargin and cyclopiazonic acid but was not affected by the absence of extracellular Ca^{2+}. Therefore, the nicorandil–induced increase in $[Ca^{2+}]_i$ may come from the extracellular space, rather than from the intracellular Ca^{2+} store. Taken together, nicorandil is considered to open ATP-sensitive K^+ channels and then to produce membrane hyperpolarization, which enhances Ca^{2+} entry by increasing the driving force.

It is well known that ATP, one of compounds released from endothelial cells (Shinozuka et al., 1994), has various pharmacological effects. In this study, nicorandil released ATP from rat caudal arterial endothelial cells. Stimulation of vascular endothelial cells with ATP evokes a rise in the level of $[Ca^{2+}]_i$ (Hallam and Pearson, 1986). It is possible that nicorandil autocrinally enhances extracellular ATP, which may increase the $[Ca^{2+}]_i$ of endothelial cells. Indeed, extracellularly supplied ATP increased $[Ca^{2+}]_i$, and this increase was inhibited by suramin, an antagonist of purinoceptors. The nicorandil-induced increase of $[Ca^{2+}]_i$, however, was not affected by suramin; therefore, it may be mediated not by the ATP released from the endothelial cells but by nicorandil.

The mechanism of ATP release stimulated by nicorandil remains to be elucidated. In rabbit and bovine ciliary epithelial cells, ATP release has been suggested to be triggered by ionomycin, which is inhibitable by the calcium/calmodulin-activated kinase II inhibitor, 1-[N,O-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) (Mitchell et al., 1998). Also, ATP is released from smooth muscle cells as a consequence of both α_1 adrenoceptor and P_2 purinoceptor activation, and Ca^{2+} plays the role of a second messenger in ATP release (Kurz et al., 1994). Increases of $[Ca^{2+}]_i$ in endothelial cells may lead to ATP release from the porcine coronary artery. We have also found that the absence of Ca^{2+} from the medium for 1 h abolishes noradrenaline-induced ATP release from the endothelium of rat caudal arteries (Nakai et al., 1996). Thus, the nicorandil-induced increase in $[Ca^{2+}]_i$ may play a crucial role in promoting the release of ATP from vessels. Clarification of the association between the release of ATP and the mobilization of intracellular Ca^{2+} is indicated for future studies.

Extracellularly released ATP, which rapidly degrades to ADP, AMP and adenosine in vascular tissues, probably by ectonucleotidases, is thought to be the source of released purines (Shinozuka et al., 1994). Thus, a high rate of ATP breakdown results in high levels of adenosine. Adenosine is known to be cardioprotective through the activation of adenosine receptors, resulting in myocardial Ca^{2+} overload via adenosine A_1 receptors, increases in coronary blood flow, and inhibition of platelet and leukocyte activity by adenosine A_2 receptors (Hori and Kitakaze, 1991). Furthermore, the most powerful cardioprotection is afforded by ischemic preconditioning (Murry et al., 1986). Therefore, adenosine has emerged as an endogenous and exogenous broad-spectrum cardioprotective agent. The opening of ATP-sensitive K^+ channels by nicorandil contributes to the activation of ectonucleotidases in ischemic preconditioning (Kitakaze et al., 1996). Thus, nicorandil may exhibit cardioprotective properties after ischemia/reperfusion injury by increasing the release of ATP from coronary vascular beds.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (2) from the Ministry of Education, Science and Culture, Japan.

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