Volume 212, number 1, 40–44 FEB 04395 February 1987

Pertussis toxin inhibits the angiotensin II and serotonin-induced rise of free cytoplasmic calcium in cultured smooth muscle cells from rat aorta

Christian Bruns and Dieter Marmé

Institut für Biologie III, University of Freiburg, Schänzlestrasse 1, 7800 Freiburg, FRG

Received 4 October 1986

Angiotensin II, serotonin and K^+-depolarization cause an increase in free cytoplasmic Ca^{2+} in cultured smooth muscle cells. The involvement of a guanine nucleotide-binding protein has been investigated by using pertussis toxin. When smooth muscle cells were pretreated with pertussis toxin angiotensin II and serotonin-induced rise of cytosolic Ca^{2+} was found to be significantly reduced whereas the Ca^{2+} influx mediated by K^+-depolarization remained unchanged. These results suggest the participation of a guanine nucleotide-binding protein in the receptor-mediated rise of intracellular Ca^{2+}.

Angiotensin II; Serotonin; intracellular Ca^{2+}; Pertussis toxin; (Smooth muscle cell)

1. INTRODUCTION

Hormonal stimuli such as angiotensin II and serotonin are able to induce contraction of vascular smooth muscle cells [1,2]. The sequence of events between hormone binding to its specific receptor and the contractile response is generally thought to consist of three steps: First, receptor-mediated activation of a phospholipase C which breaks down phosphatidylinositol 4,5-bisphosphate to give diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3) [3,4]. Second, IP_3 releases Ca^{2+} from intracellular stores, presumably from the endoplasmic reticulum, and thus increases free cytosolic Ca^{2+} [5]. Third, increased cytosolic Ca^{2+} binds to calmodulin and activates the calmodulin-dependent myosin light chain kinase [6].

The mechanism by which activation of the phospholipase C is achieved remains to be elucidated. Recent evidence obtained with other cell types than smooth muscle cells indicates, that guanine nucleotide-binding proteins (G proteins) might be involved in receptor-mediated activation of phospholipase C. In cell free systems and permeabilized cells non-hydrolysable guanine nucleotides have been found to increase the hormone-dependent generation of IP_3 (for review see [7]). Phospholipase C in a plasma membrane fraction from polymorphonuclear leukocytes was activated by the chemoattractant oligopeptide fMet-Leu-Phe and this activation could be potentiated by GTP [8]. Similar results were obtained with membrane fractions from rat pituitary (GH_3) cells where thyrotropin-releasing hormone and GTP synergistically stimulated phospholipase C activity [9]. In electrically permeabilized cells of parotid glands, non-hydrolysable GTP analogs stimulate substance P, carbachol and epinephrine-dependent formation of IP_3 [10].

Another approach to identify the participation of G proteins in the receptor-mediated activation of phospholipase C includes the use of pertussis toxin, which is known to ADP-ribosylate the α-

Correspondence address: C. Bruns, Institut für Biologie III, University of Freiburg, Schänzlestrasse 1, 7800 Freiburg, FRG

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

subunit of the G_i protein and thus blocks its action on adenylate cyclase [11]. Pertussis toxin has also been reported to inhibit the chemotactic fMet-Leu-Phe-stimulated production of IP_3 in differentiated human leukemic HL-60 cells [12]. Pretreatment of rabbit neutrophils by pertussis toxin attenuates the IP_3-mediated rise of free cytosolic Ca^{2+} induced by fMet-Leu-Phe and leukotriene B4 [13] and causes an inhibition of the polyphosphoinositide breakdown by these agents [14]. In contrast to these findings, IP_3 accumulation in chicken heart cells, induced by muscarinic receptor stimulation could not be prevented by pertussis toxin [15]. The situation seems to be even more complex because of data obtained with adrenal glomerulosa cells, where pretreatment with pertussis toxin causes inhibition of angiotensin II-induced rise of cytoplasmic Ca^{2+} without having any effect on IP_3 production [16].

A possible participation of G proteins in receptor-mediated activation of phospholipase C in smooth muscle cells has not yet been established. In the present paper we report on the inhibition by pertussis toxin of the angiotensin II and serotonin-dependent increase of free cytosolic Ca^{2+} in cultured smooth muscle cells from rat aorta. A putative role for a G protein in this process will be discussed.

2. MATERIALS AND METHODS

Angiotensin II (Ang II), elastase, serotonin (5-HT), soybean trypsin inhibitor and quin2-tetraacetoxymethyl ester (quin2-AM) were purchased from Sigma, München, FRG. Dulbecco's modified Eagle's medium (DMEM) was from Gibco, Paisley, Scotland, fetal calf serum (FCS) and collagenase were obtained from Serva, Heidelberg, FRG. Culture dishes, Falcon Primaria, were purchased from Becton Dickinson, Heidelberg, FRG. Pertussis toxin was obtained from List Biol. Lab., Campbell, USA. All other chemicals used were of analytical grade and purchased from commercial sources.

2.1. *Cell culture*

Primary cultures of vascular smooth muscle cells (VSMC) were obtained by a modification of the explantation method of Ross [17]. Briefly, thoracic aortas from male Sprague-Dawley rats were dissected free of surrounding tissue and transferred to culture dishes containing DMEM without FCS, they were cut longitudinally and the endothelium was rubbed off. The adventitia was removed with forceps to get the tunica media. The tunica media was cut into 1–2 mm^2 sections, which were placed in culture dishes containing DMEM supplemented with 10% FCS. After 14 days of incubation at 37°C in a humidified atmosphere of 5% CO_2/95% air, cultured cells migrated from the explant. After one week the migrated cells were passaged with trypsin-versene in a ratio of 1:4. The cell morphologies corresponded to typical characteristics of vascular smooth muscle cells as described by Chamley-Campbell et al. [18]. Cells of the 5th to 30th passage were used for quin2 experiments.

2.2. *Quin2 loading and measurement of $[Ca^{2+}]_i$*

The cultured smooth muscle cells were grown to reach confluency. Two replicate-plated cultures were rinsed with Hepes buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 16 mM glucose, 0.5 mM KH_2PO_4, 10 mM Hepes, 0.2% BSA and 0.2 mM $CaCl_2$, buffered to pH 7.4. Subsequently the cultures were incubated for 45 min at 37°C in Hepes buffer containing 1 mg/ml collagenase, 0.4 mg/ml soybean trypsin inhibitor and 50 U/ml elastase. Cell suspensions were obtained by gentle suction through a wide bore pipette followed by centrifugation and resuspension in Hepes buffer. Total yield was approximately $1–2 \times 10^7$ cells. Cell viability was checked by the dye exclusion method and found to be more than 95%.

Quin2, a calcium-sensitive dye, was used to monitor changes of intracellular free calcium concentration $[Ca^{2+}]$. Cell suspensions were incubated with 50 μM quin2-AM for 30 min at 37°C. At the end of the loading period, cells were washed twice and resuspended in Hepes buffer. The cell suspension was supplemented with 1 mM Ca^{2+}. 2 ml aliquots of quin2-loaded cells were transferred to cuvettes and quin2 fluorescence was detected at 37°C with a Perkin Elmer MPF-2A spectrofluorimeter equipped with a thermostated cuvette holder, stirring apparatus and chart recorder. The calibration of quin2 fluorescence and calculation of cytosolic free calcium $[Ca^{2+}]_i$ were determined as described by Tsien et al. [19].

Maximum fluorescence was obtained by permeabilizing the cells with 0.02% Triton X-100 and addition of 3 mM Ca^{2+}, minimum fluorescence was obtained by chelating calcium with 5 mM EGTA and increasing the pH above 8.3. Cells were incubated with pertussis toxin (0.5–1 μg/ml) for a total of 1 h. After 30 min of incubation the quin2-AM was added as already described. Pertussis toxin was preactivated by incubating appropriate portions in 50 mM dithiothreitol for 1 h at 25°C [20].

3. RESULTS

To provide further insights into the mechanisms of hormone-dependent, IP_3-mediated rise of free cytosolic Ca^{2+} in vascular smooth muscle cells (VSMC), we have investigated the effect of pertussis toxin on the angiotensin II and serotonin-induced increase of $[Ca^{2+}]_i$ in cultured VSMCs from rat aorta. Using the Ca^{2+}-sensitive fluorescent dye quin2 we determined the $[Ca^{2+}]_i$ in unstimulated cells to be about 175 nM (see fig.2, open control column). Upon addition of angiotensin II or serotonin, the free calcium concentration increases within 30 s up to about 370 and 320 nM, respectively (fig.1a,b). The rapid rise in $[Ca^{2+}]_i$ induced by these two hormones was only slightly decreased by adding excess of EGTA (3 mM) to chelate extracellular Ca^{2+} indicating that Ca^{2+} is mainly released from intracellular stores (not shown). Depolarization of the membrane potential achieved by the addition of 100 mM KCl results in an immediate increase of $[Ca^{2+}]_i$ to about 340 nM (fig.1c). In this case chelation of extracellular Ca^{2+} by 3 mM EGTA completely eliminated the signal suggesting that the rise in $[Ca^{2+}]_i$ is due to influx through voltage-gated Ca^{2+} channels (not shown).

Assuming that guanine nucleotide-binding proteins might be involved in receptor-dependent, IP_3-mediated release of Ca^{2+} from intracellular stores in VSMCs, we have investigated the effect of pertussis toxin on the angiotensin II and serotonin-

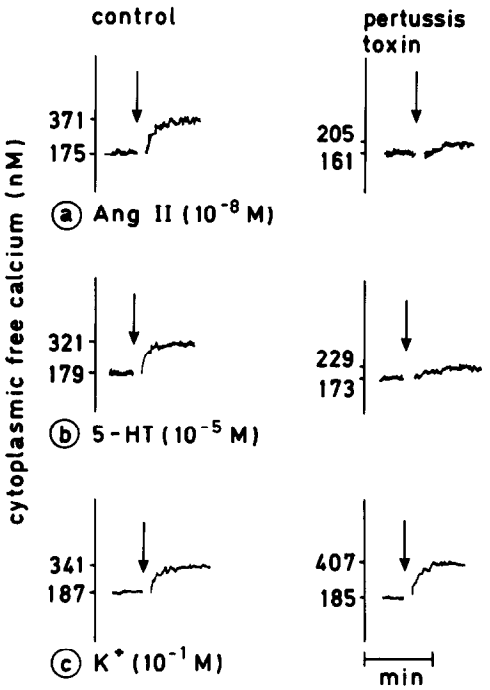

Fig.1. Quin2 measurements of $[Ca^{2+}]_i$ in cultured VSMCs. Arrows indicate the additions of 10^{-8} M Ang II (panel a), 10^{-5} M 5-HT (panel b) and 100 mM KCl (panel c). Experiments without pertussis toxin are shown as controls on the lefthand side, those after 1 h pertussis toxin pretreatment (0.5–1 μg/ml) on the right.

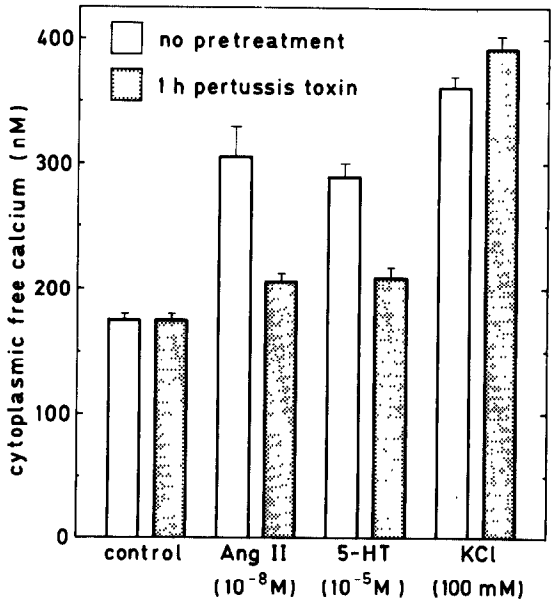

Fig.2. Effect of pretreatment for 1 h with pertussis toxin (0.5–1 μg/ml) on the increase of $[Ca^{2+}]_i$ induced by Ang II, 5-HT and KCl. Values are expressed as means ± SE of $n = 12$ (controls), $n = 5$ (Ang II), $n = 4$ (5-HT) and $n = 4$ (KCl) individual experiments.

induced rise in $[Ca^{2+}]_i$. Pretreatment of the VSMCs with 0.5–1 μg/ml pertussis toxin for 1 h at 37°C effectively inhibited the angiotensin II and serotonin-induced increase of the intracellular, free Ca^{2+} concentration (fig.1a,b). In contrast, no such inhibition was found when the rise in $[Ca^{2+}]_i$ was initiated by the addition of 100 mM KCl (fig.1c). In fig.2 we have summarized the results from several independent experiments which show that pertussis toxin has no effect on the Ca^{2+} level in unstimulated VSMCs but significantly reduces the angiotensin II and serotonin-mediated increase of $[Ca^{2+}]_i$ to almost basal levels. The K^+-dependent Ca^{2+} signal seems even to be slightly increased. However we do not regard this effect to be significant.

4. DISCUSSION

It is now generally agreed that the elevation of intracellular free Ca^{2+} caused by many hormones is mediated by IP_3 [21]. This seems to be the case also in VSMCs. Angiotensin II [3] as well as serotonin [4] have been reported to enhance the generation of IP_3. It has further been shown that IP_3 causes a Ca^{2+} release from intracellular stores of saponin-skinned primary cultured rat aortic smooth muscle cells [5]. Therefore the receptor-linked breakdown of polyphosphoinositides seems to be a major control mechanism in hormone-induced smooth muscle contraction. However, no information is available so far about the coupling of the receptor to the phospholipase C which catalyses phosphoinositol 4,5-bisphosphate hydrolysis in VSMCs. The data presented here, that pertussis toxin blocks the angiotensin II and serotonin-induced rises in cytosolic free Ca^{2+} levels, are in agreement with the assumption that a guanine nucleotide-binding protein (G protein) may participate in signal transduction. No such protein seems to be involved in voltage-gated Ca^{2+} influx through Ca^{2+} channels.

Recently it has been reported that pertussis toxin is an effective inhibitor of both angiotensin II-induced breakdown of phosphatidylinositol 4,5-bisphosphate and increase of $[Ca^{2+}]_i$ in rat renal mesangial cells [22]. In contrast to this report and to our data it has been shown that pertussis toxin does not affect angiotensin II-dependent formation of IP_3 and intracellular Ca^{2+} release in adrenal glomerulosa cells but does inhibit angiotensin II-mediated Ca^{2+} influx [16]. In hepatocytes the EGF-induced rise in $[Ca^{2+}]_i$ was blocked by pertussis toxin but not the angiotensin II-stimulated Ca^{2+} increase [23].

These data suggest that the angiotensin II receptor coupling to phospholipase C is different in different cell types and does not necessarily include a G-protein like component which can be blocked by pertussis toxin as we have observed for VSMCs. Thus the situation seems to be more complex as in the case of receptor-dependent inhibition of adenylate cyclase [11].

In addition to G proteins being involved in the activation of phospholipase C it has also been reported that GTP is able to enhance Ca^{2+} release from a rat liver microsomal fraction caused by IP_3 [24]. It could well be that pertussis toxin interferes with this process and thus inhibits Ca^{2+} release from intracellular organelles without interfering with the receptor-mediated activation of phospholipase C.

Based on the data presented here obtained with VSMCs we cannot discriminate between the two possibilities of pertussis toxin blocking the formation of IP_3 and thus inhibiting the rise in intracellular Ca^{2+} or pertussis toxin blocking the IP_3-mediated Ca^{2+} release from intracellular stores. Appropriate experiments are being performed to unravel the molecular mechanism by which pertussis toxin can inhibit the angiotensin II and serotonin-induced increase of free cytosolic Ca^{2+} in vascular smooth muscle cells.

ACKNOWLEDGEMENTS

We would like to thank Mr K. Gössl for preparing the figures and Mrs R. Zimmermann for typing the manuscript.

REFERENCES

[1] Lalanne, C., Mironneau, C., Mironneau, J. and Savineau, J.P. (1984) Br. J. Pharmac. 81, 317–326.
[2] Maggi, C.A., Manzini, S. and Meli, A. (1983) Eur. J. Pharmacol. 94, 251–260.
[3] Alexander, R.W., Brock, T.A., Gimbrone, M.A. jr and Rittenhouse, S.E. (1985) Hypertension 7, 447–451.

[4] Doyle, V.M., Creba, J.A., Rüegg, U.T. and Hoyer, D. (1986) Naunyn-Schmiedeberg's Arch. Pharmacol. 333, 98–103.
[5] Yamamoto, H. and Van Breemen, C. (1985) Biochem. Biophys. Res. Commun. 130, 270–274.
[6] Walsh, M.P. (1985) in: Calcium and Cell Physiology (Marmé, D. ed.) pp.170–203, Springer, Berlin.
[7] Litosch, I. and Fain, J.N. (1986) Life Sci. 39, 187–194.
[8] Smith, C.D., Cox, C.C. and Snyderman, R. (1986) Science 232, 97–100.
[9] Straub, R.E. and Gershengorn, M.C. (1986) J. Biol. Chem. 261, 2712–2717.
[10] Taylor, C.W., Merritt, J.E., Putney, J.W. jr and Rubin, R.P. (1986) Biochem. Biophys. Res. Commun. 136, 362–368.
[11] Gilman, A.G. (1984) Cell 86, 577–579.
[12] Brandt, S.J., Dougherty, R.W., Lapetina, E.G. and Niedel, J.E. (1985) Proc. Natl. Acad. Sci. USA 82, 3277–3280.
[13] Molski, T.F.P., Naccache, P.H., Marsh, M.L., Kermode, J., Becker, E.L. and Sha'afi, R.I. (1984) Biochem. Biophys. Res. Commun. 124, 644–650.
[14] Holian, A. (1986) FEBS Lett. 201, 15–19.
[15] Masters, S.B., Martin, M.W., Harden, T.K. and Brown, J.H. (1985) Biochem. J. 227, 933–937.
[16] Kojima, I., Shibata, H. and Ogata, E. (1986) FEBS Lett. 204, 347–351.
[17] Ross, R. (1971) J. Cell Biol. 50, 172–186.
[18] Chamley-Campbell, J., Campbell, G.R. and Ross, R. (1979) Physiol. Rev. 59, 1–61.
[19] Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) J. Cell Biol. 94, 325–334.
[20] Wong, S.K.F., Martin, B.R. and Tolkovsky, A.M. (1985) Biochem. J. 232, 191–197.
[21] Hokin, L.E. (1985) Annu. Rev. Biochem. 54, 205–235.
[22] Pfeilschifter, J. and Bauer, C. (1986) Biochem. J. 236, 289–294.
[23] Johnson, R.M., Connelly, P.A., Sisk, R.B., Pobiner, B.F., Hewlett, E.L. and Garrison, J.C. (1986) Proc. Natl. Acad. Sci. USA 83, 2032–2036.
[24] Dawson, A.P., Comerford, J.G. and Fulton, D.V. (1986) Biochem. J. 234, 311–315.