Activation of phosphatidylinositol synthesis by different agonists in a primary culture of smooth muscle cells grown on collagen microcarriers

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Regulation of inositol phosphate synthesis was examined in a primary culture of vascular smooth muscle cells grown on collagen-coated microcarriers. In the presence of LiCl (10 mM), four agonists [serotonin, angiotensin, (arginine) vasopressin and noradrenaline] were found to stimulate the formation of inositol phosphates in a dose-dependent manner. All agonists were found to have identical and additive effects on the time course of inositol phosphate formation. Therefore, our primary cell culture technique was proved to give smooth muscle cells suitable for the study of modulation of phosphoinositide metabolism in response to physiological effectors.

(Aortic myocyte) *Cell culture* *Muscle contraction* *Agonist* *Inositide metabolism*

1. INTRODUCTION

The rise of intracellular Ca^{2+} concentration has been shown to play a crucial role in the regulation of vascular smooth muscle contraction. It appears that the mechanism by which some hormones induce an increase in intracellular Ca^{2+} concentration involves the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol [1]. IP_3 has been shown in several systems to be able to release Ca^{2+} from the endoplasmic reticulum [2,3].

Abbreviations: AII, angiotensin II; AVP, (Arg)-vasopressin; NA, noradrenaline; 5-HT, 5-hydroxytryptamine; IP, *myo*-inositol phosphate; IP_3, *myo*-inositol 1,4,5-trisphosphate; PBS, phosphate-buffered saline; PIP_2, phosphatidylinositol 4,5-bisphosphate; $d(CH_2)_5$-AVP, [1-β-mercapto-β-(β'-cyclopentamethylene-propionic acid)-8-arginine] vasopressin

In the past few years, vascular smooth muscle cells from either primary cultures [4–6] or stable cell lines [7–9] have been used to study the biochemical coupling between regulatory hormone receptors and the main physiological response, i.e. contraction [10–13]. Smooth muscle cells from rat aorta respond to and have receptors for the hormones such as vasopressin and angiotensin and for neurotransmitters such as noradrenaline and serotonin. These responses have been shown to be mediated by phosphoinositide breakdown in aorta [10,14–16] but none of the primary cell cultures described so far responded to all 4 agonists. In fact, vascular smooth muscle cells seem to lose some of their initial properties during the exponential phase of proliferation, thereby limiting their usefulness in studies on vasoactive hormone action [17–19].

We show here that aortic smooth muscle cells cultured on collagen-coated microcarriers retain their ability to respond to the 4 agonists by an in-

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crease in inositol phosphate formation. Some properties of these stimulations are also discussed.

2. MATERIALS AND METHODS

2.1. *Materials*

Hormones ((Arg) AVP, AII, NA, 5-HT) were from Sigma (St. Louis, MO). *myo*-2-[^{3}H]Inositol (16.5 Ci/mmol) was obtained from New England Nuclear. Culture medium and supplements were purchased from Biomerieux and fetal calf serum from Gibco. Collagen-coated microcarriers were from IBF (Société Chimique Pointet Girard). All other chemicals were of analytical grade.

2.2. *Preparation of aortic myocytes*

Vascular smooth muscle cells were prepared from male adult rat aortas as in [6] with some modifications. Briefly, male Wistar rats (12 weeks old) were killed by cervical dislocation. Aortas were dissected and digested for 30 min in a collagenase solution (type II, 300 U/ml). Adventitia and intima were removed and the release of free vascular smooth muscle cells was achieved through incubation of the remaining media for 90 min in a collagenase-elastase solution. Myocytes were then cultured with continuous stirring for 13–14 days on collagen-coated microcarriers in modified Eagles medium (MEM) supplemented with 10^{-5} M L-proline, 1.5×10^{-6} M ascorbic acid and 5% fetal calf serum.

2.3. *Measurement of phosphoinositide hydrolysis*

In intact cells, labeling of phosphoinositides was performed by incubation for 2 days in the medium previously described, complemented with 2.5 μCi/ml *myo*-[^{3}H]inositol. Labeled cells were washed 3 times with PBS buffer before use and then sampled (approx. 200000 cells per sample). Each sample (890 μl) was incubated with 10 mM LiCl in PBS buffer to inhibit inositol-1-phosphate phosphatase and to allow accumulation of inositol phosphates [20,21]. Agonists were added and cells incubated at 37°C for various times. The reaction was stopped by addition of 400 μl of 10% perchloric acid, and ^{3}H-labeled inositol phosphates extracted, resolved from other labeled material and quantified as described [22].

3. RESULTS AND DISCUSSION

As shown in fig.1, the 4 main regulatory agents of rat aortic contraction were able to stimulate IP accumulation when tested for 45 min in the presence of LiCl. Hormone concentrations were 10^{-4} M for 5-HT, 10^{-7} M for AII and AVP, 10^{-5} M for NA and accumulation of IP was stimulated 5.63 ± 0.76, 5.28 ± 0.5, 2.94 ± 0.35 and 1.78 ± 0.3-fold, respectively (mean ± SE of 4 experiments). The effect of noradrenaline (NA) is likely to be mediated by an α_1-adrenergic effect since a similar effect was observed in the presence of 10^{-5} M phenylephrine, moreover there was no detectable stimulation of the synthesis of IP by 10^{-5} M clonidine, a specific α_2-agonist. All responses were abolished in the presence of the specific antagonist of each hormone (fig.2).

Dose-response curves are presented in fig.3. Half-maximal activations were obtained in the presence of 5.3×10^{-6} M 5-HT, 3.5×10^{-9} M AII,

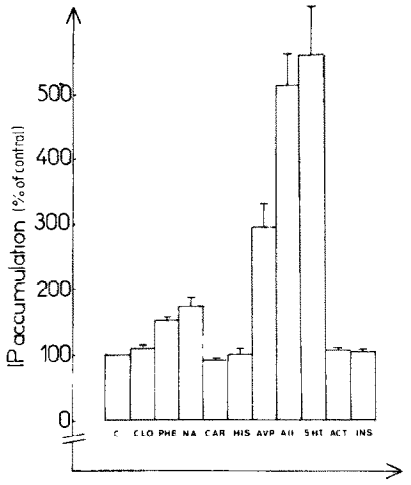

Fig.1. Screening of different hormonal stimulation for IP accumulation in the presence of 10 mM LiCl. Data shown are means, expressed as percentage of the maximum response induced by each agonist, of triplicate determinations and are representative of 3 such studies. C, control; CLO, clonidine (10^{-5} M); Phe, phenylephrine (10^{-5} M); NA, noradrenaline (10^{-5} M); CAR, carbachol (10^{-4} M); His, histamine (10^{-4} M); AVP, (Arg) vasopressin (10^{-7} M); AII, angiotensin II (10^{-7} M); 5-HT, 5-hydroxytryptamine (10^{-4} M); ACTH, adrenocorticotropic hormones (4–10) (10^{-6} M); INS, insulin (10^{-7} M).

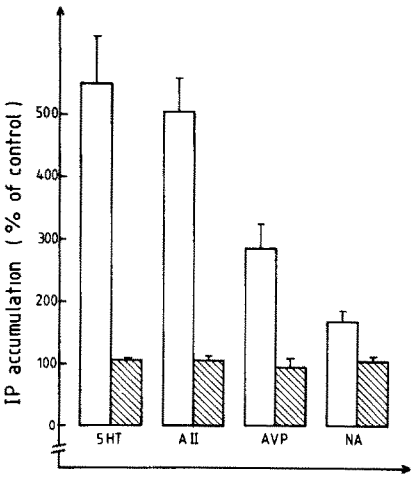

Fig.2. Antagonism of IP accumulation elicited by the 4 stimulations. Open bars: responses obtained with 10^{-4} M 5-HT, 10^{-7} M AII, 10^{-7} M AVP and 10^{-5} M NA. Hatched bars: responses obtained in the presence of the following antagonists – ketanserin (10^{-5} M), for 5-HT stimulation; (Sar, Ile) Ang (10^{-6} M), for AII stimulation; $d(CH_2)_5AVP$ (4×10^{-7} M), for AVP stimulation; prazosin (10^{-6} M), for NA stimulation. Antagonists were applied 10 min prior to addition of the agonists. Results are means ± SE of a determination performed in triplicate.

4.5×10^{-9} M AVP and 2.3×10^{-7} M NA. These values are very close to those reported to produce an increase in phosphatidylinositol metabolism and/or contraction in aorta tissues or cells [10,14–16]. The time course of accumulation of the 3 inositol phosphates was measured in the presence of concentrations of agonists able to give maximal activation of IP formation. Fig.4 shows that all agonists exhibit very similar behaviour with respect to their kinetics.

The results presented in fig.5 indicate that phosphatidylinositol-bisphosphate phosphodies-

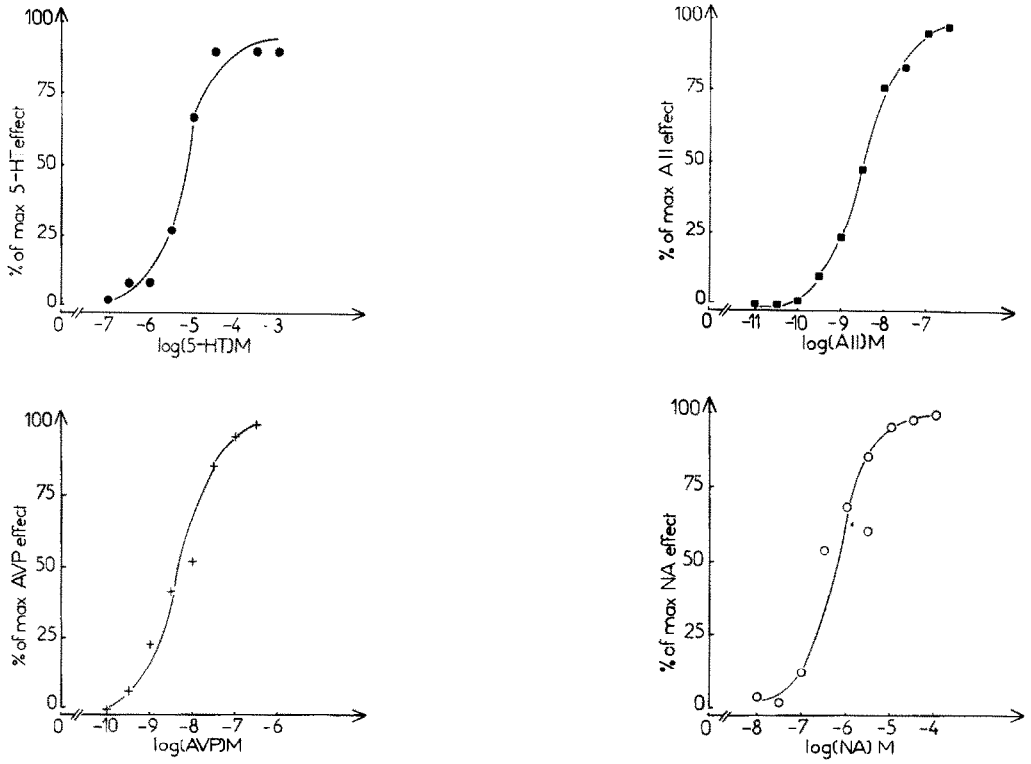

Fig.3. Dose-response curves to (AII, (Arg)-AVP, 5-HT, NA) for accumulation of IP. Data shown are the means, expressed as a percentage of the maximum response induced by each agonist, of triplicate determinations and are representative of 3 such studies.

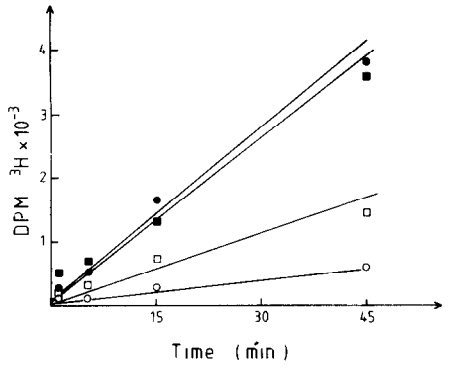

Fig.4. Time course of the effect of the different agonists on IP accumulation in the presence of 10 mM LiCl. Each point is the mean of a determination performed in triplicate and representative of 3 such studies. (●) 10^{-4} M 5-HT, (■) 10^{-7} M AII, (□) 10^{-7} M AVP, (○) 10^{-5} M NA.

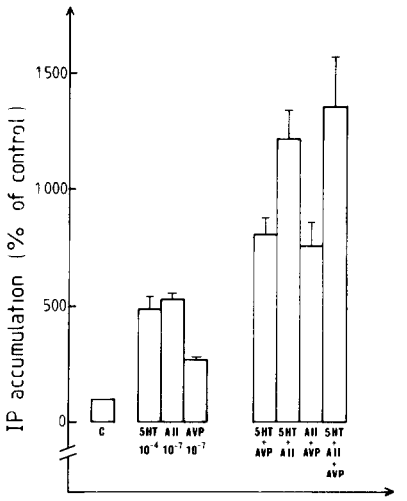

Fig.5. Additivity of the different agonists in the presence of 10 mM LiCl. Concentrations of agonists: 5-HT, 10^{-4} M; AII, 10^{-7} M; AVP, 10^{-7} M. Each bar indicates a mean ± SE of 3 determinations.

terase is not maximally activated, irrespective of the hormone used for stimulation. This figure shows an additivity of the respective hormonal stimulations, each hormone being used at a concentration giving a maximal independent stimulation. These results indicate that part of but not all phosphodiesterase activity might be activated through single hormone stimulation. Therefore, it seems that the rate of IP_3 formation is limited only by the number of phosphodiesterase molecules activated upon hormone binding. The additivity tests were made at 45 min when the rate of IP_3 accumulation was still in the steady-state phase (fig.4). It is possible, however, that in the presence of 2 or 3 hormones, steady-state conditions no longer exist, thereby explaining the close but not accurate additivity of hormonal stimulations. Another explanation could be the presence in the culture of different types of myocytes which could express the 4 receptors differently.

Our preliminary experiments indicate that vascular smooth muscle cells prepared as described above and grown on collagen-coated microcarriers still present responsiveness to hormones and properties of vascular material. All 4 receptors tested were functional and their activation upon binding of the respective hormones led to activation of IP formation, probably through activation of the PIP_2 phosphodiesterase. Culture on microbeads allowed us to obtain large amounts of material suitable for biochemicals as well as pharmacological studies. The first studies reported herein indicate that these cells are very similar to in situ cells. The precise characterization of each receptor and the analyses of their similarities with intact artery, in terms of hormone binding, potency in the triggering of muscle contraction will be further investigated.

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