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MODULATION OF PROSTACYCLIN BIOSYNTHESIS BY CALCIUM ENTRY BLOCKERS AND EXTRACELLULAR CALCIUM

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Abstract—The influence of variations in the availability of extracellular Ca^{2+} and of Ca^{2+}-entry blockers on prostacyclin production by mesothelial cells in culture was studied. The Ca^{2+}-entry blockers nifedipine and verapamil suppressed the basal, as well as the thrombin-, bradykinin-, and ionophore A23187-stimulated biosynthesis by about 50–60%, but high concentrations were required and the inhibition was never complete. Basal prostacyclin formation was unaffected by a Ca^{2+}-poor buffer, but showed 50% reduction in the Ca^{2+}-free buffer. Although the thrombin-stimulated prostacyclin formation was not significantly influenced by a Ca^{2+}-poor or a Ca^{2+}-free buffer, prostacyclin release stimulated by A23187 and bradykinin was diminished in the presence of these modified incubation media; the reduction of bradykinin stimulated biosynthesis was rather small (30%). These results suggest that the Ca^{2+} from intracellular stores is sufficient for half maximal stimulation of the phospholipases involved in the biosynthetic pathway of prostacyclin and that—depending on the nature of the stimulus—different phospholipases are activated with varying requirements for free Ca^{2+}.

Release of arachidonic acid from membrane phospholipids is considered as the rate-limiting step for prostaglandin biosynthesis [1, 2]. This release occurs after membrane perturbation or stimulation of specific receptors, whereby phospholipases of the A2 type are activated, although phospholipases of the C type might also be involved [3, 4]. For the activity of phospholipases, the presence of Ca^{2+}-ions is required [5, 6].

In order to study the possible involvement of intra- and extracellular Ca^{2+} in the prostaglandin biosynthesis, we investigated how the prostacyclin production in cultured mesothelial cells is influenced by various Ca^{2+}-entry blockers and by variation of the Ca^{2+} concentration in the incubation medium. Cultures of mesothelial cells were chosen since they have the same embryonic origin as vascular endothelial cells and, like endothelial cells, synthesize mainly prostacyclin from the endogenous cyclooxygenase substrate arachidonic acid [7, 8].

MATERIALS AND METHODS

Materials. Plastic materials, media and serum for tissue culture were obtained from Gibco Europe (Paisley, Scotland). Routine laboratory products and reagents were of analytical grade and purchased from Merck (Darmstadt, F.R.G.). Ionophore A23187, bradykinin and bovine serum albumin were from Sigma (St. Louis, MO). Thrombin (Topostasine) was obtained from Roche (Belgium) and verapamil from Knoll (Ludwigshafen, F.R.G.). EGTA (ethylene glycol-bis-(2-aminoethyl)-tetraacetic acid) was purchased from Ciba Geigy (Basel, Switzerland). Nifedipine and flunarizine were gifts from respectively Bayer (Belgium) and Janssen Pharmaceutica (Beerse, Belgium).

Cell culture and incubation. Rabbit mesothelial cells were isolated after a brief collagenase digestion of the omentum maius and cultured as described previously [8]. The cells were grown in Dulbecco's MEM, containing 15% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycine and 0.25 μg/ml amphotericin B, enriched with 1% non-essential amino acids solution (Gibco-Europe).

For incubation, cells (second subculture) were grown in Petri dishes (35 × 10 mm). When the cells had reached confluence, the culture medium was replaced by a physiological, Hepes buffered solution (150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 5 mM glucose, 10 mM Hepes, pH 7.5) and the cells were preincubated for 10 min at 37°, in the presence of the possible inhibitors (nifedipine, verapamil and flunarizine). After this preincubation the buffer solutions were replaced by fresh solutions, containing the same inhibitors, and the monolayers were incubated for 10 min at 37°, in the presence of one of the different stimuli of prostacyclin formation. Variations in the extracellular Ca^{2+} concentration were studied by preincubating and incubating the cells in either normal physiological salt solution (1.8 mM Ca^{2+}), or Ca^{2+}-poor, or Ca^{2+}-free buffer solutions. These solutions consisted of the same physiological Hepes buffered salt solution from which $CaCl_2$ was omitted (Ca^{2+}-poor) or where the remaining traces of Ca^{2+}-ions were chelated with 1 mM EGTA (Ca^{2+}-free). The incubation reactions were stopped by

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removal of the incubation fluid, which was used for the assay of prostacyclin, and by addition of 0.3 ml ice-cold 5% trichloroacetic acid solution (TCA) to the remaining cell monolayer. The precipitated cell protein was scraped off with a policeman, the procedure was repeated and the combined TCA mixture was centrifuged (10 min, 10,000 *g*). The protein pellet was dissolved in 0.1 N NaOH and used for the determination of the cellular protein content, according to Lowry *et al.* [9].

Influence of extracellular Ca^{2+} *concentrations.* To study the influence of variations in the extracellular Ca^{2+} concentration on the basal and stimulated prostacyclin production, incubations were carried out in Ca^{2+}-poor and Ca^{2+}-free buffer solutions. The Ca^{2+}-poor buffer still contained traces of Ca^{2+} present as impurity of the other salts or the distilled water, or originating from the incubated cells. In the Ca^{2+}-free buffer, these traces of Ca^{2+} ions were chelated by the addition of 1 mM EGTA.

The effects of both buffers on the basal, as well as on the stimulated prostacyclin production are given in Fig. 1. This figure shows for each stimulus the relative prostacyclin biosynthesis as compared to the production by the mesothelial cells in the normal $CaCl_2$ containing salt solution. Both bradykinin- and A23187-stimulated prostacyclin production were diminished in either buffer with a tendency towards a larger reduction in the Ca^{2+}-free solution; however, this tendency was statistically not significant. In the Ca^{2+}-free buffer, bradykinin- and A23187-stimulated prostacyclin production decreased with respectively 31 ± 14% and 53 ± 9%. Basal prostacyclin biosynthesis was significantly reduced only in the Ca^{2+}-free buffer (with 52 ± 8%) whereas thrombin-stimulated production was never significantly influenced by the reduced availability of extracellular Ca^{2+}.

Radioimmunoassay of 6-oxo-$PGF_{1\alpha}$. Prostacyclin was measured by a specific radioimmunoassay of 6-oxo-$PGF_{1\alpha}$, as described previously [8], and expressed as pmol prostacyclin/mg cell protein. None of the substances tested (i.e. the stimuli, Ca^{2+} entry blockers) interfered with the radioimmunoassay. The experiments were repeated with 3 to 4 cell lines. Because of some variation in prostacyclin production between the different cell lines, the percent inhibition with respect to the paired control was calculated on each occasion.

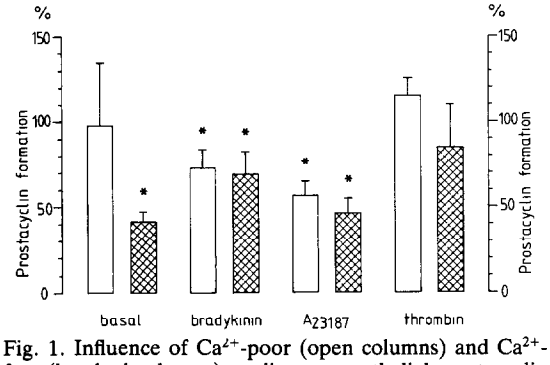

Fig. 1. Influence of Ca^{2+}-poor (open columns) and Ca^{2+}-free (hatched columns) media on mesothelial prostacyclin biosynthesis under basal conditions, and in monolayers stimulated with 1 μM bradykinin, 10 μM A23187 or 10 units thrombin/ml. The results are given as percent of the paired control (i.e. stimulus in the presence of 1.8 mM Ca^{2+}) and are given as mean ± S.E.M. of five different cell lines. The control prostacyclin biosynthesis was 41 ± 16 pmol/mg under basal conditions, 420 ± 89 pmol/mg in the presence of bradykinin, 958 ± 221 pmol/mg in the presence of A23187 and 212 ± 39 pmol/mg in the presence of thrombin. *$P < 0.05$, significantly different from 100%, two-tailed Student *t*-test.

RESULTS

Effect of the slow channel Ca^{2+} *entry blockers*

As shown in Fig. 2, the prostacyclin production in mesothelial cells in culture was dose-dependently stimulated by A23187, thrombin and bradykinin. Further experiments were carried out with 10 U/ml thrombin, 10 μM A23187 and 1 μM bradykinin, all concentrations which produced a submaximum stimulation of prostacyclin biosynthesis. Depolarizing media, in which part of the NaCl was replaced by 10, 25 or 50 mM KCl, failed to stimulate the basal prostacyclin production. The prostacyclin biosynthesis stimulated by bradykinin or thrombin was not significantly affected by the presence of 50 mM KCl in the buffer (Results not shown). In order to investigate the effect of different types of Ca^{2+} entry blockers on basal as well as stimulated prostacyclin formation, the dihydropyridine derivative nifedipine, the papaverine derivative verapamil and the piperazine derivative flunarizine were used. Nifedipine (100 μM) inhibited basal prostacyclin production by 68 ± 7% (mean ± S.E.M., N = 4, $P < 0.05$, Student's *t*-test). The dose–response curves for the inhibition of the prostacyclin production, stimulated by bradykinin, A23187 and thrombin are shown in Fig. 3 (a–c). The inhibition exerted by 100 μM nifedipine was 65 ± 6% after stimulation of the prostacyclin production with A23187 and was 55 ± 9% and 48 ± 16% after stimulation with bradykinin and thrombin respectively. Verapamil (100 μM) decreased the A232187-, bradykinin- and thrombin-stimulated formation by respectively 61 ± 11%, 61 ± 10% and 68 ± 9% (mean ± S.E.M., three experiments, $P < 0.05$, Student's *t*-test) (Fig. 3). Flunarizine (100 μM) on the contrary, failed to

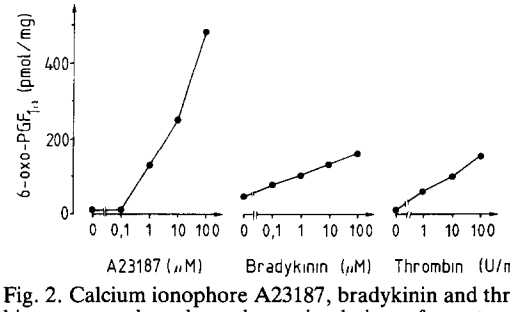

Fig. 2. Calcium ionophore A23187, bradykinin and thrombin cause a dose-dependent stimulation of prostacyclin biosynthesis in monolayers of cultured rabbit mesothelial cells. Prostacyclin was measured by radioimmunoassay of the stable metabolite 6-oxo-PGF_1, and its formation was expressed per mg cellular protein. Examples of single experiments are shown. The monolayers stimulated with A23187 and thrombin originated from the same cell line.

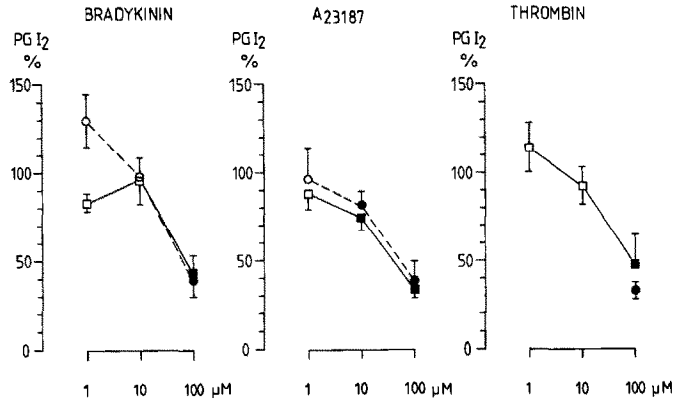

Fig. 3. Effect of nifedipine (—□—) and verapamil (—○—) on prostacyclin biosynthesis in cultured mesothelial cells. Prostacyclin biosynthesis was stimulated by 1 μM bradykinin, 10 μM A23187 and 10 units thrombin/ml. The results were expressed as percent of the paired control (i.e. stimulus without calcium entry blockers) and given as mean ± S.E.M. of four (nifedipine) or five (verapamil) different cell lines. Closed symbols are significantly different from 100% ($P < 0.05$, Student's *t*-test, two tailed).

inhibit the basal or the stimulated prostacyclin production. It even caused a statistically significant enhancement of the stimulation of the prostacyclin biosynthesis with thrombin or bradykinin (increase respectively 75 ± 24 and 43 ± 11%, mean ± S.E.M. in five cell lines).

DISCUSSION

The Ca^{2+}-ionophore A23187, bradykinin and thrombin stimulate prostacyclin biosynthesis in cultured mesothelial cells. The mechanism of action of A23187 is based on a transport of Ca^{2+}-ions through biological membranes [10]. Also, bradykinin and thrombin have been shown to stimulate a Ca^{2+} influx in the cell [11, 12]. The stimulation of the prostacyclin production by these agonists, as well as the basal formation could be inhibited by two "slow channel" Ca^{2+} entry blockers. With the exception of thrombin, the prostacyclin biosynthesis in response to these agonists was also reduced in a Ca^{2+}-free buffer. These results clearly indicate that an influx of external Ca^{2+} may contribute to the biosynthesis of prostacyclin. The inhibition of A23187-induced prostacyclin formation by verapamil and nifedipine was a little surprising, but a similar effect has been reported previously [13].

Basal prostacyclin production was diminished by about 65% by eliminating Ca^{2+} from the extracellular medium, as well as by adding the "slow channel" antagonist nifedipine. However, no inhibition of the basal prostacyclin formation was found in the Ca^{2+}-poor buffer, where trace amounts of Ca^{2+} were still present. Thus, intracellular Ca^{2+} was, under basal circumstances, able to provide for the phospholipase activity necessary to maintain one third of the prostacyclin production, whereas trace amounts of extracellular Ca^{2+} were sufficient to maintain basal production on its normal level.

In a Ca^{2+}-free solution, A23187-stimulated prostacyclin formation was reduced with only 50% and in the presence of extracellular Ca^{2+}, the inhibition by the slow channel antagonists nifedipine and verapamil never exceeded 60%. Thus apparently, A23187 was able to release Ca^{2+} from intracellular binding or storage sites, so that about half of the stimulation of the prostacyclin production was maintained. Moreover, it has been shown in cultured endothelial cells, that the stimulation of prostacyclin biosynthesis by A23187 can be completely blocked with 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8) [14], a putative antagonist of intracellular Ca^{2+} mobilization [15, 16]. These results confirm the fact that A23187 can transport Ca^{2+} from extracellular origin as well as from intracellular storages sites. The effect of both fluxes can be blocked with TMB-8 by suppressing either acylhydrolase [14] or cyclo-oxygenase activity [17], whereas nifedipine and verapamil only block the influx of extracellular Ca^{2+}.

The fact that the effects of A23187 can be partly blocked with Ca^{2+} entry blockers, could indicate that A23187 facilitates Ca^{2+} transport via Ca^{2+} gates already existing, instead of creating new channels. On the other hand, the mechanism of action of the Ca^{2+} entry blockers is not yet completely elucidated: dihydropyridines (like nifedipine) are thought to interact with specific receptor sites, on or near the Ca^{2+} gates, whereas verapamil, and other papaverine derivatives, might interfere with the cell membrane in a non-specific manner [18].

In the Ca^{2+}-free buffer solution, stimulation of the prostacyclin production with bradykinin was inhibited with 30%, while the stimulation caused by thrombin was not significantly affected. On the contrary, in the presence of extracellular Ca^{2+}, nifedipine and verapamil caused a more substantial inhibition of thrombin as well as bradykinin stimulated prostacyclin formation, with about 50% (nifedipine) and 65% (verapamil) respectively. Thus the stimulation of prostacyclin biosynthesis by thrombin and bradykinin also depends on extracellular as well as intracellular Ca^{2+}-sources. The different results obtained in the Ca^{2+}-free buffer on the one hand and with the Ca^{2+} entry blockers on the other hand, might indicate that these Ca^{2+} entry blockers act

partly on intracellularly bound Ca^{2+}. At present we have no explanation for the discrepancies with the results obtained in bovine aortic endothelial cells in culture, in which the prostacyclin production stimulated by bradykinin was completely blocked with 100 μM nifedipine, 100 μM verapamil or 5 mM EGTA [19], whereas in our experiments the inhibition of the prostacyclin biosynthesis was never complete.

This inconsistency could be due to differences between the cell types studied (e.g. species, origin and confluency of cells) or to the strength of the stimulus used. However, our data are consistent with results obtained with rat superior mesenteric artery smooth muscle cells in culture. The vasopressin evoked prostacyclin biosynthesis of these cells was inhibited by a maximum of only 50–60% at extracellular calcium concentrations of 10^{-5} M or lower, whereas cinnarizine and nifedipine had no significant effects [17].

The fact that flunarizine showed no inhibitory action, was not completely unexpected. In vascular smooth muscle, flunarizine is able to inhibit the Ca^{2+} influx stimulated by vasoactive substances, but is inactive when Ca^{2+} channels are opened through intrinsic changes in membrane permeability. Moreover, flunarizine is unable to antagonize Ca^{2+} stimulated positive inotropic effects on myocardial tissue [20, 21].

Our experiments show that the release of intracellularly bound Ca^{2+} seems to be sufficient to maintain about 50% of the basal and stimulated prostacyclin production, which makes it unlikely that pharmacological use of Ca^{2+} entry blockers will greatly influence mesothelial prostacyclin production. This view is further supported by the fact that in cultured mesothelial cells as well as in endothelial cells, the effects of nifedipine and verapamil only become visible when relatively high concentrations are used. Furthermore, it has recently been reported that nifedipine does not alter the increased release of prostacyclin from the acutely ischemic canine myocardium [22].

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