JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 1999, 50, 4, 639—652

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CROSS TALK BETWEEN NO AND CYCLIC NUCLEOTIDE PHOSPHODIESTERASES IN THE MODULATION OF SIGNAL TRANSDUCTION IN BLOOD VESSEL

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An increase in cAMP and/or cGMP induces vasodilation which could be potentiated by endothelium or NO-donors. Cyclic nucleotide phosphodiesterases (PDE) are differently distributed in vascular tissues. cAMP hydrolyzing PDE isozymes in endothelial cells are represented by PDE2 (cGMP stimulated-PDE) and PDE4 (cGMP insensitive-PDE), whereas in smooth muscle cells PDE3 (cGMP inhibited-PDE) and PDE4 are present. To investigate the role of NO in vasodilation induced by PDE inhibitors, we studied the effects of PDE3- or PDE4-inhibitor alone and their combination on cyclic nucleotide levels, on relaxation of precontracted aorta and on protein kinase implication. Furthermore, the direct effect of dinitrosyl iron complex (DNIC) was studied on purified recombinant PDE4B.
The results show that: 1) in endothelial cells PDE4 inhibition may up-regulate basal production of NO, this effect being potentiated by PDE2 inhibition; 2) in smooth muscle cGMP produced by NO inhibits PDE3 and increases cAMP level allowing PDE4 to participate in vascular contraction; 3) protein kinase G mediates the relaxing effects of PDE3 or PDE4 inhibition. 4) DNIC inhibits non competitively PDE4B indicating a direct effect of NO on PDE4 which could explain an additive vasodilatory effect of NO. A direct and a cGMP related cross-talk between NO and cAMP-PDEs, may participate into the vasomodulation mediated by cAMP activation of protein kinase G.

Key words: *cGMP, cAMP, NO, DNIC, endothelial cell, smooth muscle, PDE2, PDE3, PDE4, Protein kinase G, relaxation, rat aorta.*

INTRODUCTION

Although it is well established that cAMP and cGMP induce vasodilatation, the respective role in vasodilatation of each nucleotide is not clearly understood. In the same way, vasodilatation is dependent of NO produced by endothelial cells which cover smooth muscle tissue (1, 2). By stimulating cytosolic guanylyl cyclase, NO increases cGMP level, and

consequently potentiates relaxation. Moreover, cGMP differently modulates the various cyclic nucleotide phosphodiesterase (PDE) isozymes which hydrolyse cAMP (3). cGMP activates PDE2 whereas inhibits PDE3. Furthermore, PDE4 is insensitive to cGMP. Since these PDEs are differently distributed in endothelial and vascular smooth muscle cells (4, 5) the increase in cGMP level, resulting from NO production, may induce tissue differences in the regulation of cyclic nucleotide metabolism and vasomodulation.

To study the interaction of NO in the participation of vasodilation induced by PDE inhibitors, we studied the effect of PDE3- or PDE4- inhibitor and their combination on: 1) the variation of cAMP and cGMP content in cultured endothelial cells under conditions modulating NO production; 2) the variation of cAMP and cGMP content in aorta without and with functional endothelium; 3) the relaxation of precontracted aorta without and with endothelium; 4) the participation of protein kinases in the relaxation. The interaction of dinitrosyl iron complex (DNIC) was studied on purified recombinant PDE4B.

This study shows reciprocal regulations between NO and cyclic nucleotides suggesting a cross-talk between PDE3 and PDE4, depending on endogenous cGMP, which participates in the vasomodulation mediated by protein kinase G in response to cAMP increase.

MATERIALS AND METHODS

Assay of cyclic nucleotide levels in endothelial cells and in rat aorta

Cyclic nucleotide levels were determined by radioimmunoassay in confluent cultured bovine aortic endothelial cells (BAEC) (6) and rat aorta without or with functional endothelium (7) treated for 5 minutes with PDE inhibitors in the presence of 10 μM indomethacin without or with NO modulators. Drug vehicle groups were incubated with dimethyl sulfoxide (DMSO) 1%, the solvent of PDE inhibitors. Results are expressed in fmol/μg DNA.

Measurement of aortic relaxation

Rat (Wistar 350—450 g) aorta rings were mounted under 2 g of resting tension in organ baths containing 20 ml Krebs solution. Studies of PDE inhibitors and protein kinase inhibitors on noradrenaline or $PGF_{2\alpha}$ precontracted aorta were done as reported (8, 9)

Phosphodiesterases studies

Phosphodiesterase activity, IC_{50} and K_i values on purified recombinant PDE4B, kindly given by Dr M. Perry (Celltech, UK) were determined by using ^{3}H-cAMP (10). To characterize PDE4 Western blot was performed with 50 μg protein from cultured human umbilical vein endothelial cells (HUVEC) and bovine aortic media using a specific PDE4B antibody kindly provided by Dr M. Conti (11) (K118 used at 1/2000 dilution).

Statistical analysis

Statistical comparisons were performed according to analysis of variance (ANOVA) and Student's t-test. Results are expressed as the mean ± s.e. mean. In figures vertical bars indicates: ±s.e.mean. Differences were considered significant when $P<0.05$, $P<0.01$, $P<0.005$, $P<0.001$.

MODULATION OF CYCLIC NUCLEOTIDE CONTENTS IN VASCULAR ENDOTHELIAL CELLS

Bovine aortic endothelial cell (BAEC) cytosol obtained from confluent culture contains only PDE2 and PDE4 (12, 13). To investigate their respective role in the regulation of NO production in non-stimulated BAEC, we measured cyclic nucleotide levels in cultured treated for 5 min with specific PDE inhibitors. Since endothelial cells do not contain PDE3, cilostamide (a specific inhibitor of PDE3, $K_i = 0.042$ μM (5) inhibits in this study preferentially PDE2 ($K_i = 15$ μM, 5). Therefore cilostamide (50 μM) was used as PDE2 inhibitor and rolipram (20 μM) was used as PDE4 inhibitor, $K_i = 0.76$ μM (5), alone or together, in indomethacin-exposed BAEC cobblestone culture in the presence and absence of the NO/cGMP pathway modulators: a NO-synthase inhibitor (300 μM N^G-nitro-L-arginine methyl ester, L-NAME) and the NO-synthase substrate (100 μM L-arginine).

Table 1 shows that in control endothelial cell cultures, cAMP level was 5 fold greater than cGMP level. Neither exposition to the substrate nor to the inhibitor of NO synthase significantly changed cyclic nucleotide levels as compared to control cells, indicating that in basal conditions, the low cGMP level is not sufficient to detect a modulation of NO/cGMP pathway.

Table 1. Effect of NO modulators on cAMP and cGMP contents in cultured bovine aortic endothelial cells.

Treatment	cAMP (fmol/μg DNA)	cGMP (fmol/μg DNA)
Control	254 ± 41	49 ± 10
L-arginine (100 μM)	298 ± 55	37 ± 4
L-NAME (300 μM)	349 ± 76	40 ± 15

Results represent the means ± s.e. mean of values obtained in triplicate from three different cultures (6).

In BAEC either without or with modulator of NO/cGMP pathway, PDE2 inhibitor (cilostamide, 50 μM) or PDE4 inhibitor (rolipram, 20 μM) alone did not increase cAMP level. However, the combination of phosphodiesterase inhibitors potently increased cAMP levels by 858 ± 12% ($P<0.001$), 1286 ± 17% ($P>0.001$) and by 1130 ± 18% ($P>0.001$) for control, L-arginine and L-NAME treated cells, respectively.

In the same samples, no significant effect of PDE2 and PDE4 inhibitors (alone or in combination on cGMP contents were observed in control and L-NAME treated cultures. Surprisingly, in the presence of L-arginine cGMP content was significantly increased by rolipram (by 42±2% compared to control cells, $P<0.05$), which specifically inhibits cAMP hydrolysis, and not significantly by cilostamide alone. Furthermore, combination of these phosphodiesterase inhibitors resulted in a marked increase in cGMP level (to 153±3%, $P<0.001$). The association potentiated the effects of cilostamide alone ($P<0,05$) and the effects of rolipram alone ($P< 0.001$). None of these effects were observed in the presence of L-arginine+ L-NAME (6).

MODULATION OF CYCLIC NUCLEOTIDE CONTENTS IN RAT AORTA

Effect of endothelium and NO modulating agents on basal cyclic nucleotide contents

Cyclic nucleotide contents were determined in rat aorta without and with endothelium under modulators of NO contents (*Table 2*).

Table 2. Cyclic nucleotide contents in rat aorta and effects of NO modulators.

	cAMP	cGMP
	(fmol/μg DNA)	
Aorta without endothelium		
Control	23.3±2.5 (n = 8)	6.4±1.9 (n = 6)
SIN-1 (10 μM) +SOD (100 U/mol)	30±3.5 (n = 6)	88±19*** (n = 6)
Aorta with endothelium		
Control	39.5±3.1^{+++} (n = 10)	14±0.3^{+++} (n = 9)
L-NAME (30 μM)	36.9±4.0 (n = 8)	6.8±1.0*** (n = 10)

Results are the mean±s.e. mean of n experiments. *** $p < 0.001$ in comparison with their control. $^{+++}$ $p < 0.001$ in comparison with aorta without endothelium (7).

In the control aorta without endothelium, cAMP content was about 4 fold higher than cGMP content. As expected, addition of SIN-1 in combination with SOD markedly increased cGMP content by 14 fold without modifying significantly cAMP content.

In the control aorta with endothelium, cAMP content and cGMP were significantly higher than in the absence of endothelium (219% and 170%, respectively). As expected, addition of L-NAME (30 μM), an inhibitor of NO-synthase, restored cGMP content to the cGMP content of rat aorta without endothelium, without modifying cAMP content.

Effect PDE3 and PDE4 inhibitors on cyclic nucleotide contents in aorta under endothelium and NO modulations

Neither SK&F 94120 (30 μM), PDE3 inhibitor $K_i = 5.2$ μM (5), nor rolipram (30 μM), alone or combined, significantly modified cGMP content in aorta without or with endothelium whatever NO modulations.

In control aorta without endothelium (*Fig. 1A*), PDE3 as PDE4 inhibitor significantly elevated cAMP contents (1.7 and 2 fold respectively). Furthermore, combination of rolipram with a low concentration of SK&F 94120 potentiated the effect of rolipram (1.7 fold). Pretreatment with SIN-1 plus SOD (*Fig. 2B*) markedly enhanced the cAMP content induced by rolipram (6 fold), although cAMP content was not modified by SK&F 94120. Moreover, combination of rolipram with a low concentration of SK&94120, did no more potentiate this increase.

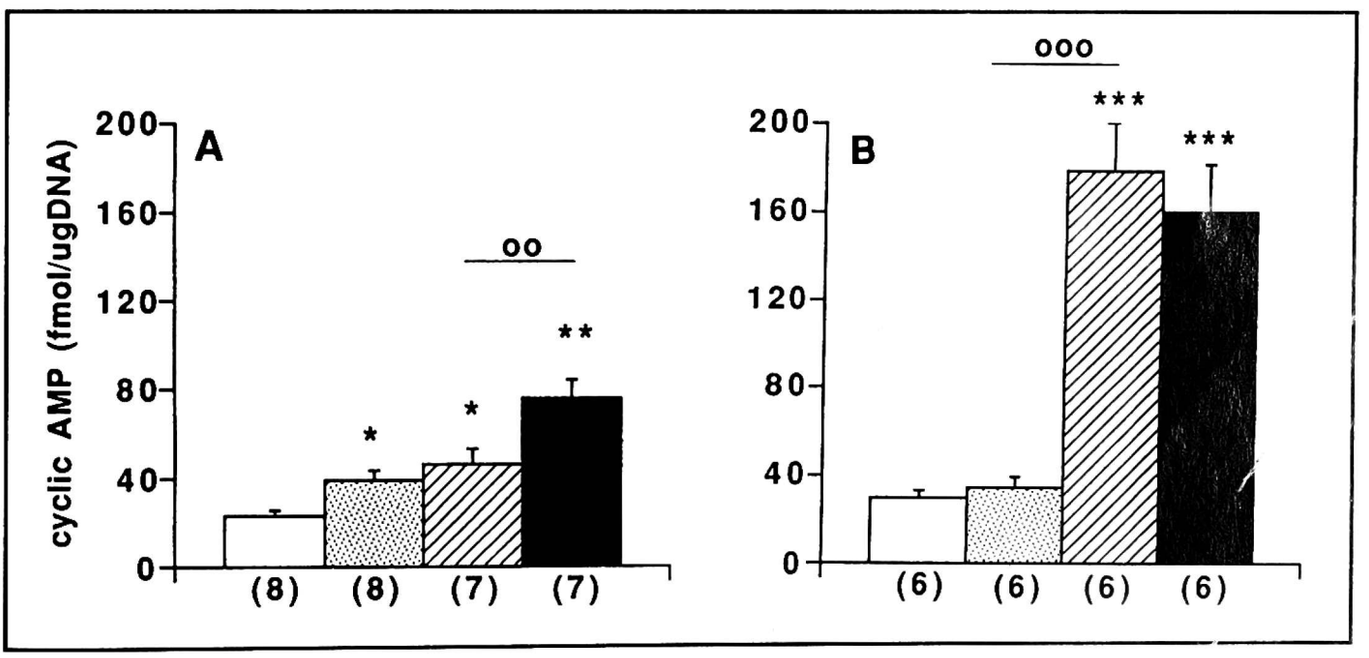

Fig. 1. Effects of PDE3 and PDE4 inhibitors on cAMP content in control aorta without endothelium (A) or treated with SIN-1 plus SOD (B). The rings were incubated in the presence of drug vehicle (0.1% DMSO, open columns), PDE3 inhibitor (30 μM SK&F 94120, stippled columns), PDE4 inhibitor (30 μM rolipram, hatched columns) or with combination of 5 μM SK&F 94120 (a concentration which does not increase cAMP content) and 30 μM rolipram (solid columns). The results are expressed as the mean of 6 to 8 experiments (number indicated under the columns) ± s.e.mean. $P<0.05$, $P<0.01$, $P<0.001$ in comparison with aorta incubated with drug vehicle (first column), °° $P<0.01$, °°° $P<0.001$ in comparison with the previous adjacent column (7).

In aorta with endothelium, only rolipram, alone or in combination with SK&F 94120 were able to significantly increase cAMP contents in control (1.7 and 2.6 fold) and L-NAME treated aorta (1.7 and 2.7, not illustrated) (7).

RAT AORTA CONTRACTION

To study the participation of PDE3 and PDE4 in the vasomodulation, the relaxing effects of PDE3 and PDE4 inhibitors was investigated on noradrenaline precontracted rat aorta without and with functional endothelium and under the modulation of NO donor, or agents increasing cAMP or cGMP levels.

Effects of PDE3 inhibitors

The relaxing effect of selective PDE3 inhibitors: indolidan, CI 930, milrinone and SK&F 94120 were studied by performing concentration-relaxation curves with increasing concentrations of PDE3 inhibitors on aorta without and with endothelium (*Fig. 2A*). As reported

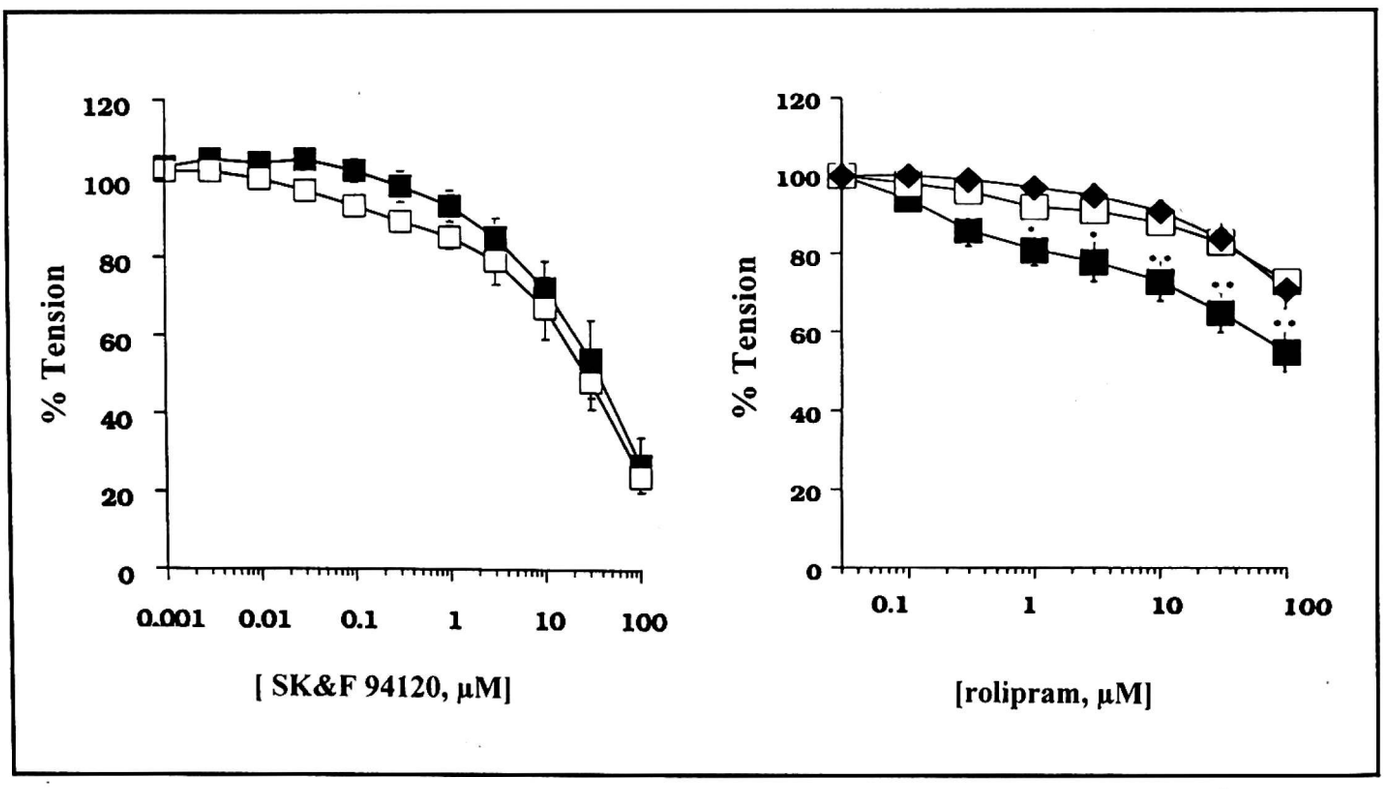

Fig. 2. Relaxation of noradrenaline-precontracted rat aortic rings by PDE3 inhibitor (SK&F 94120) and PDE4 inhibitor (rolipram) without (open square) and with functional endothelium in absence (filled square) or presence of N^G-monomethyl-L-arginine (black diamond) (n = 8). Significant differences were determined by ANOVA (*P < 0.05, **P < 0.01, compared without functional endothelium) followed by Newman-Keuls'test.

on *Table 3*, all the PDE3 inhibitors assayed induced a relaxing effect with EC_{50} values which were not significantly modified by the presence of endothelium. Moreover, in the presence of endothelium the relaxing effects of PDE3 inhibitors were insensitive to L-arginine (1mM), the substrate of NO synthase, or to L-NMMA (300 μM), an inhibitor of NO synthase, indicating that PDE3 inhibitors are endothelium-independent relaxing agents (not illustrated). The different EC_{50} values on relaxation were correlated to their IC_{50} on isolated rat aorta PDE3 (8) suggesting that relaxation is due to PDE inhibition.

Table 3. Relaxing potency of PDE3 inhibitors on noradrenaline-precontracted (1 μM) rat aortic rings (EC_{50}, μM).

Inhibitors	EC_{50} on relaxation (μM)	
	With functional endothelium	Without functional endothelium
Indolidan	3.4±0.9 (n = 6)	3.1±0.9 (n = 6)
Milrinone	5.7±1.0 (n = 6)	9.0±3.0 (n = 6)
CI 930	7.8±3.0 (n = 8)	5.1±2.0 (n = 8)
SK&F 94120	14.7±4.0 (n = 6)	14.2±5.0 (n = 6)

Results represent the mean±s.e. mean of n rings. (8).

Effects of PDE4 inhibitors

As shown in *Fig. 2B*, PDE4 inhibitors, rolipram and denbufylline (not illustrated), did not produce any significant relaxation, except at very high concentrations, in rat aortic rings without functional endothelium. In the presence of functional endothelium, PDE4 inhibitors caused a concentration-depent relaxation which was reversed by L-NMMA to the concentration-curves obtained in absence of endothelium. Further addition of L-arginine restored relaxation. Therefore, PDE4 inhibitors behaved as endothelium-dependent relaxing agents (8).

Combination of PDE4 inhibitors with other cyclic nucleotide elevating agents

To investigate the mechanism of the participation of endothelium in the relaxing effects of PDE4 inhibitors, their relaxing effects (EC_{50}) were studied in the presence of low concentrations (which relaxed precontracted aorta by about 10%) of compounds able to increase levels of either cAMP (isoprenaline, a β-adrenoceptor agonist; forskolin, an adenylyl cyclase activator; and milrinone, a PDE3 inhibitor) or cGMP (sodium nitroprusside, SNP, a soluble activator of guanylyl cyclase) on aorta without and with functional endothelium (*Table 4*).

Table 4. Influence of cyclic nucleotide elevating agents on the potency (EC_{50}, μM) of rolipram and denbufylline to relax prostanglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-precontracted rat aorta rings, without or with endothelium (E).

	Denbufylline		Rolipram	
	Without E	With E	Without E	With E
Control	NS	30.2±9.0	NS	152±18
Isoprenaline (0.1 μM)	NS	1.7±0.6	NS	13±5
Forskolin (0.3 μM)	NS	1.4±0.6	NS	6.7±3.0
Milrinone (0.03 μM)	1.7±0.3	0.4±0.02	12.2±2.6	6.7±3.0
SNP (0.001 μM)	12.3±4.0	0.7±0.3	124±26	1.2±0.5

Results are expressed as mean of 6 experiments±s.e. mean. In order to obtain the same level of contraction prior the addition of denbufylline or rolipram, the aortic rings were stimulated either by 1 μM $PGF_{2\alpha}$ (control rings) or by 2 μM $PGF_{2\alpha}$ (rings exposed to cyclic nucleotide elevating agents at a concentration reducing contraction by 10%). NS = not significant ($EC_{50} \gg 200$ μM). (8).

In the absence of endothelium, isoprenaline (0.1 μM) as forskolin (0.3 μM) did not significantly modify the concentration-curve of rolipram or denbufylline. However, milrinone (0.030 μM) markedly potentiated the PDE4 inhibitor relaxing effects. SNP potentiated to a lesser extend (10 fold) the effect of rolipram and denbufylline. The EC_{50} values obtained for rolipram and denbufylline in the presence of SNP were similar to those obtained for the control curves in the presence of endothelium.

With functional endothelium, isoprenaline, forskolin, milrinone and SNP markedly potentiated the relaxing effects of rolipram and denbufylline, their EC_{50} values obtained in the presence of SNP being similar to the IC_{50} values determined on isolated PDE4 from rat aorta (8).

PARTICIPATION OF PROTEIN KINASES IN THE RELAXATION INDUCED BY cAMP ELEVATING AGENTS

To investigate the involvement of cyclic nucleotide dependent protein kinases in cAMP-induced vasodilatation the effect of protein kinase A (PKA) inhibitor (H89, 1 μM) and protein kinase G (PKG) inhibitor (Rp-8-Br-cGMPS, 10 μM) were studied on the concentration-relaxation curves of isoprenaline (β-adrenoceptor agonist), SK&F 94120 (PDE3 inhibitor) and rolipram (PDE4 inhibitor) in rat aortic rings with endothelium.

As expected, the concentration-relaxation curve of isoprenaline was significantly modified by pretreatment with PKA inhibitor, and was unchanged with PKG inhibitor. However the decrease in relaxation due to PKA inhibitor was only obtained with isoprenaline concentrations lower than 1 μM (not illustrated).

Conversely, concentration-relaxation curves of SK&F 94120 (*Fig. 3A*) and rolipram (*Fig. 3B*) were significantly modified by pretreatment with PKG inhibitor and not by PKA inhibitor, suggesting that PKG activation mediates the relaxation induced by PDE3- and PDE4-inhibitors (9).

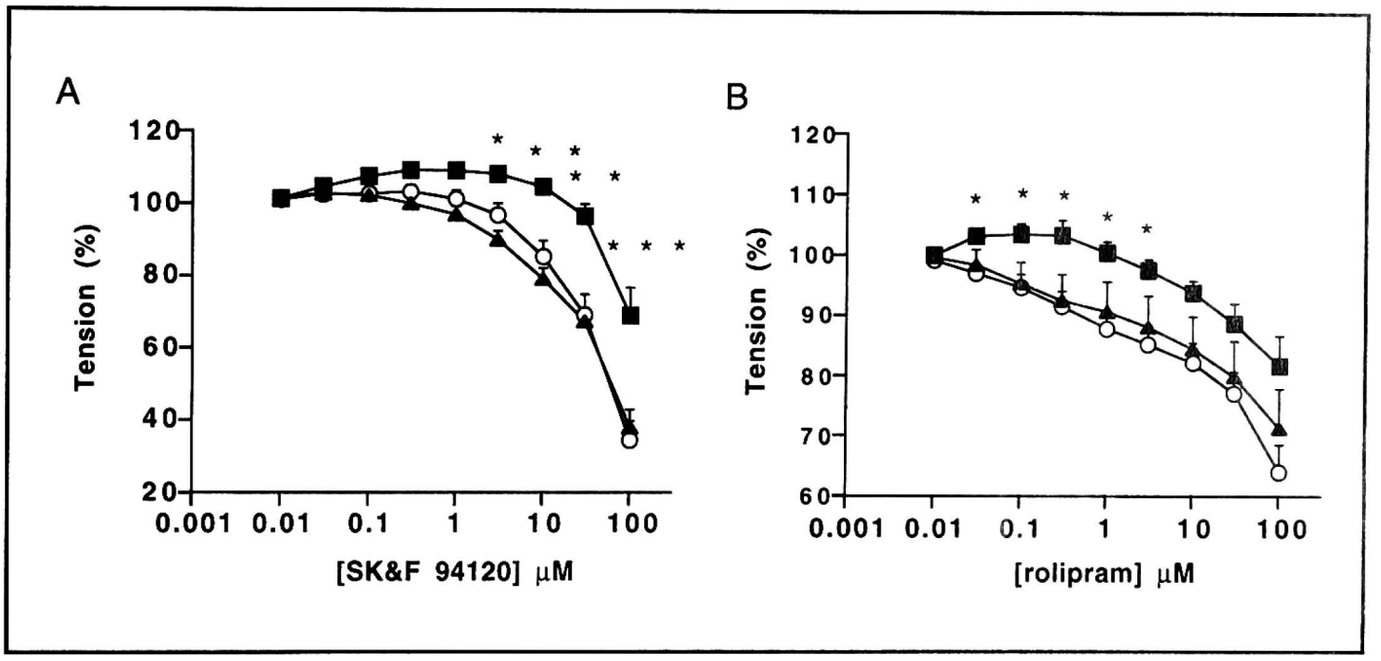

Fig. 3. Relaxation of noradrenaline-precontracted rat aortic rings by PDE3 inhibitor (SK&F 94120) and PDE4 inhibitor (rolipram) with functional endothelium. Control aorta (open circle), aortic rings pretreated with PKA inhibitor, H89 1 μM (filled triangle), aortic rings pretreated with PKG inhibitor , Rp-8Br-cGMPs 10 μM (filled square). The results are expressed as the mean of 6 to 10 experiments . Significant differences were determined by ANOVA (*$P<0.05$, **$P<0.01$ between rings treated or not with protein kinase inhibitors).

EFFECT OF DINITROSYL IRON COMPLEX (DNIC) ON RECOMBINANT PDE4B

We have detected by Western blot under denaturing conditions two variants of PDE4B subtype in endothelial cells (75 kDa and 90 kDA) and in smooth muscle cells (56 kDa and 90kDa). Since it was reported that NO could interact directly with protein (14) and that this process could participate to vasodilatation (15), we questioned whether PDE could be a direct target of NO. We investigated the effect of DNIC on PDE4B.

DNIC inhibited 1 μM cAMP hydrolysis by PDE4B with an IC_{50} value of 0.85 ± 0.06 μM. This inhibitory effect was protected by 30 μM cysteine increasing the IC_{50} value to 8.5 ± 1.1 μM, whereas it was potentiated by DETC 100 μM (DNIC $IC_{50} = 0.088 \pm 0.009$ μM). At the used concentration neither cysteine nor DETC interacted with PDE4B. Lineweaver-Burk plot revealed

that DNIC behaved as a non competitive inhibitor with an apparent K_i value of 6 ± 1 μM, suggesting that it does not interact with the catalytic site; this is in agreement with data showing that cAMP does not protect PDE4B from the inhibitory effect of DNIC.

DISCUSSION

The present study clearly shows that depending on the presence of functional endothelium in blood vessel, cAMP hydrolysing enzymes, which are differently distributed in endothelial cells and smooth muscle cells, modulate vascular tone in various manners, allowing a fine tuning of signal transduction in blood vessel.

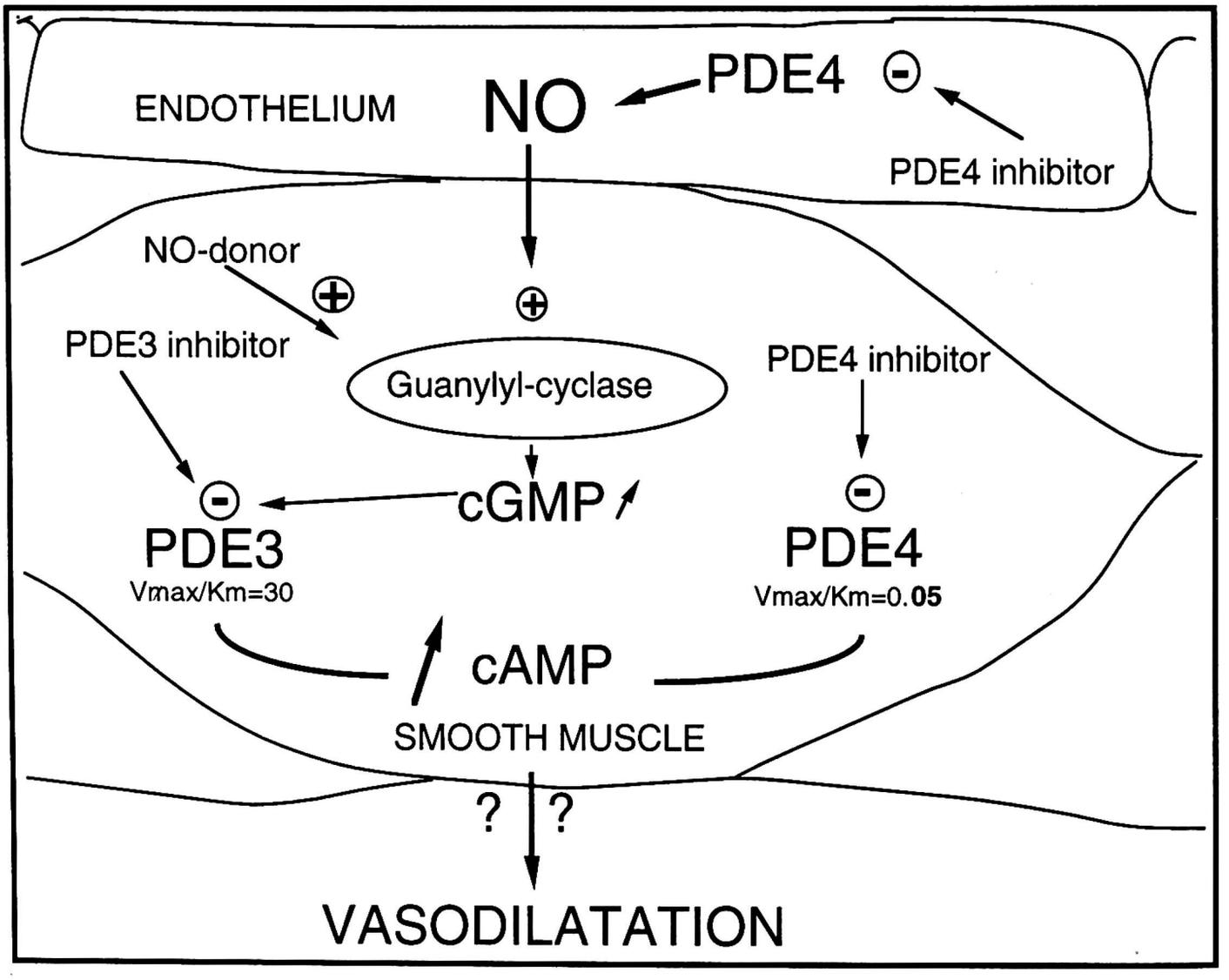

Fig. 4. Regulation by NO of the participation of PDE3 and PDE4 in vasomodulation. 1) In absence of endothelium, smooth muscle PDE3, due to its high V_{max} and low K_m values for cAMP, represents the first metabolizing enzyme able to control low level of cAMP. 2) Its inhibition, either by PDE3 specific inhibitors or by cGMP related to NO donor, increases cAMP content allowing therefore participation of PDE4 in cAMP metabolism. 3) In the presence of functional endothelium, endothelial NO, by stimulating guanylyl cyclase, increases cGMP which inhibits smooth muscle PDE3 increasing cAMP content to a sufficient level allowing PDE4 activity. 4) In endothelial cells inhibition of PDE4 induces increase of NO which can consequently modulate PDE3 and vasodilate.

In endothelial cells in which only PDE2 and PDE4 are present, specific inhibition of PDE4 induced a significant cGMP increase. Since this effect was observed only in the presence of L-arginine (NOS substrate), it is suggested that PDE4 inhibition may upregulate endothelial NOS. This shows for the first time that PDE inhibition is able to increase NO in non activated endothelial cells.

In aorta smooth muscle both PDE3 and PDE4 hydrolyse cAMP, whereas PDE2 is absent. The direct (PDE3 inhibitors) or NO mediated (related to cGMP increase) PDE3 inhibition allows the participation of PDE4 in vasomodulation (*Fig. 4*). Since PDE3 has a higher V_{max} and a lower K_m value for cAMP hydrolysis in comparison with PDE4 (3), the first PDE metabolising cAMP in vascular tissue is PDE3. Since NO-related cGMP and PDE3 inhibitors interact both at the PDE3 catalytic site, PDE3 inhibitors are endothelium-independent relaxing agents able to modulate low level of cAMP, whereas PDE4 inhibitors are able to modulate only higher level of cyclic AMP resulting of PDE3 inhibition. This may explain our observation that PDE4 inhibitors are endothelium-dependent relaxing agents (8).

It has been assumed that activation of cyclic AMP dependent protein kinase (PKA) could represent one of the mechanism by which an increase of cAMP could induce vasorelaxation (16). However, several studies have suggested that PKA is not the sole mediator of cyclic AMP-dependent vasorelaxation and an important role has been attributed to cyclic GMP-dependent protein kinase (PKG) in the vasorelaxation (17—19). Therefore we investigated the implication of PKA and PKG in the relaxing effect of PDE3 and PDE4 inhibitors.

The results clearly show that activation of PKG is implicated in the response to PDE3 or PDE4 inhibitors (*Fig. 5*), whereas PKA is implicated is the vasorelaxing effect of low concentration of β-agonist or combination of PDE3 and PDE4 inhibitors (9). Furthermore PKG activation decreases the ATP -induced transient increase in $[Ca^{2+}]_i$ (9). This cross-talk between cAMP and PKG could be explained by colocalization of PDE3 and PDE4 with PKG, their specific inhibition inducing a localized high increase in cAMP able to activate PKG, since we showed that PDE3 and PDE4 are associated to various membrane structures of the cell (10) (*Fig. 5*).

Studies performed with DNIC, a compound which favours the interaction NO-protein (14), show that DNIC inhibits non competitively PDE4B isoform. This PDE subtype is present in endothelial as well as in smooth muscle cells. It is hypothetized that NO resulting from upregulation of NOS is able to inhibit vascular PDE4 and therefore modulate positively vasodilatation. In vivo and ex vivo investigations would be necessary to check this hypothesis (*Fig. 5*).

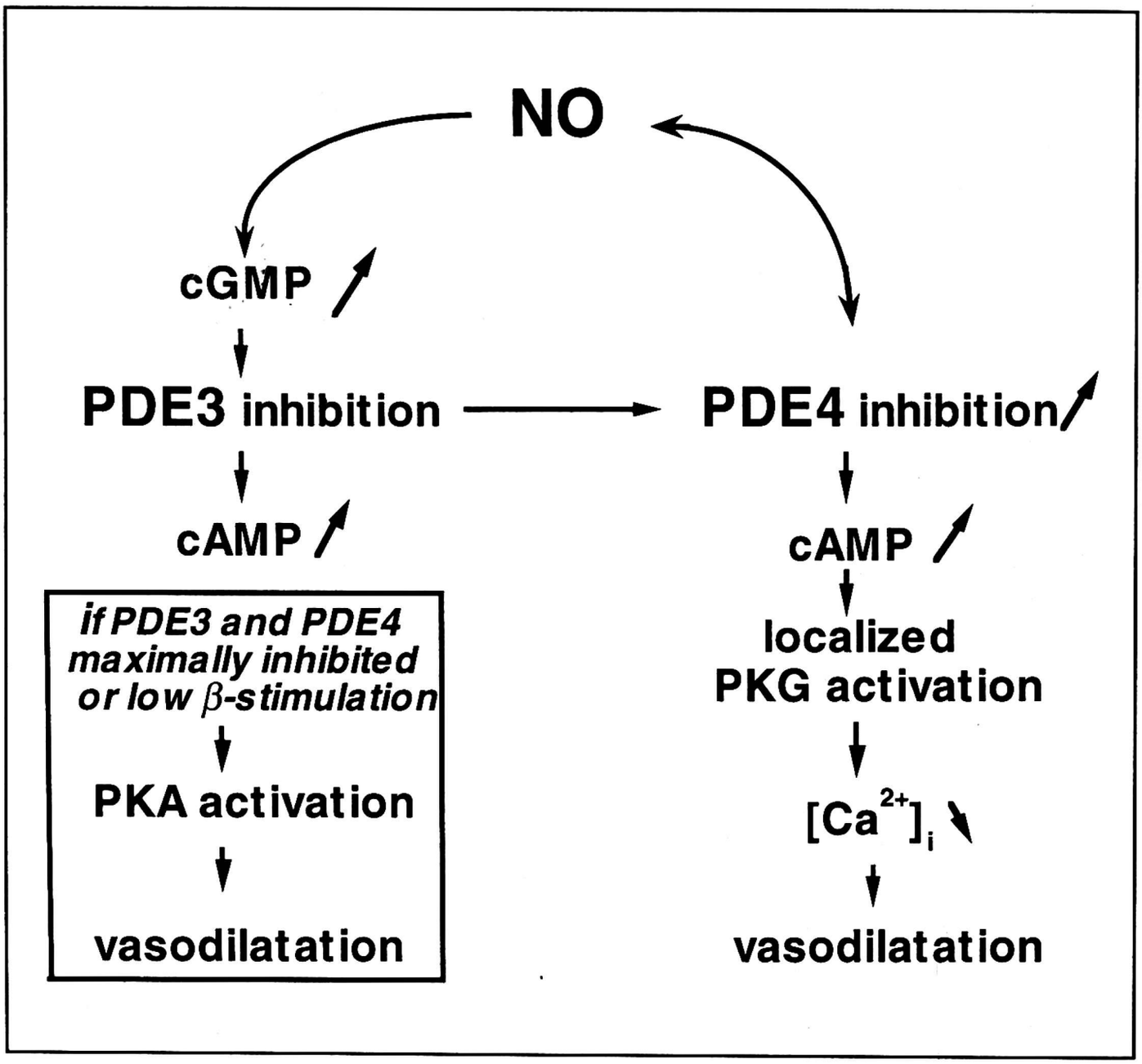

Fig. 5. Scheme of hypothetical compartments of cyclic AMP pathway in blood vessel. When PDE3 or PDE4 alone are inhibited there is a localized increase of cAMP which allows activation of PKG, decrease of intracellular calcium and vasodilatation. Hower when cAMP is increased either by β-agonist (<0.3μM) or by combination of PDE3 and PDE4 inhibitors, PKA is activated. The degree of accumulation of cAMP diffusing in the cellplays an important role in the involvement of PKA or PKG.

Together, it appears that PDE3 and PDE4 in blood vessel can play an important role in vasodilatation modulated by NO either by indirect inhibition of PDE3 or by direct inhibition of PDE4. Also, PDE4 is able to modulate NO in endothelial cells.

Acknowledgment: This work was supported by the "Centre National de la Recherche Scientifique" and by the "Institut National de la Santé et de la Recherche Médicale". We would like to thank Dr. M. Conti and Dr M Perry for kindly providing us with the PDE4B antibody and recombinant PDE4B, respectively.

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Received: July 21, 1999
Accepted: September 21, 1999

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