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EFFECTS OF ADENOSINE 3′ : 5′-MONOPHOSPHATE AND GUANOSINE 3′ : 5′-MONOPHOSPHATE ON CALCIUM UPTAKE AND PHOSPHORYLATION IN MEMBRANE FRACTIONS OF VASCULAR SMOOTH MUSCLE

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Summary

The effects of adenosine 3′ : 5′-monophosphate (cyclic AMP), guanosine 3′ : 5′-monophosphate (cyclic GMP) and exogenous protein kinase on Ca uptake and membrane phosphorylation were studied in subcellular fractions of vascular smooth muscle from rabbit aorta. Two functionally distinct fractions were separated on a continuous sucrose gradient: a light fraction enriched in endoplasmic reticulum (fraction E) and a heavier fraction containing mainly plasma membranes (fraction P).

While cyclic AMP and cyclic GMP had no effect on Ca uptake in the absence of oxalate, both cyclic nucleotides inhibited the rate of oxalate-activated Ca uptake when used at concentrations higher than 10^{-5} M. The addition of bovine heart protein kinase to either fraction produced an increase in the rate of oxalate-activated Ca uptake which was further augmented by cyclic AMP. Cyclic GMP caused smaller stimulations of protein kinase-catalyzed Ca uptake than cyclic AMP.

Mg-dependent phosphorylation, attributable to endogenous protein kinase(s), was inhibited in fraction E by low concentrations (10^{-8} M) of both cyclic AMP and cyclic GMP. In fraction P, an inhibition by cyclic AMP occurred also at a concentration of 10^{-8} M, while with cyclic AMP a concentration of 10^{-5} M was required for a similar inhibition. Bovine heart protein kinase stimulated the phosphorylation of the membrane fractions much more than Ca uptake. In fraction E, in the presence of bovine protein kinase, both cyclic AMP and cyclic GMP stimulated phosphorylation up to 200%. Under these conditions, no stimulation was observed in fraction P.

These results are compatible with the hypothesis that in vascular smooth muscle soluble rather than particulate protein kinases are involved in the regulation of intracellular Ca concentration.

Abbreviation: EGTA, ethyleneglycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

Introduction

In a variety of smooth muscles, including vascular smooth muscle, cyclic AMP has been proposed as the mediator of the relaxation in response to β-adrenoceptor stimulation (for reviews see refs. 1—3). Studies utilizing α- and β-adrenoceptor stimulating and blocking agents have shown that activation of vascular β-adrenoceptors generally results in both an elevation of the cyclic AMP content and a relaxation of the vascular tissue, whereas stimulation of the α-adrenoceptors is followed by a decrease in cyclic AMP content and contraction. However, the results of several studies do not fit into this general concept [3].

As to cyclic GMP, it has been suggested that it is involved in the contractile response of smooth muscle to several agonists [2]. However, recent studies [4,5] showing an increase in cyclic GMP content in various smooth muscles under the influence of relaxing drugs have cast some doubts on the validity of a functional antagonism between cyclic AMP and cyclic GMP in smooth muscle. For the explanation of the intracellular effects of cyclic AMP on contraction in smooth muscle, a similar sequence of events as that put forward for cardiac muscle [6] can be proposed: cyclic AMP acts on protein kinase(s) which are responsible for the phosphorylation of an intracellular Ca accumulating- and/or a plasma membrane-located Ca extruding system. Modification in the degree of phosphorylation of these systems would in turn alter their ability to accumulate or to pump Ca. As a final consequence, Ca^{2+} concentration in the cytoplasm, which is directly responsible for the activation of the contractile proteins, is altered. There are only few publications dealing with the influence of cyclic nucleotides on the handling of Ca by subcellular fractions of vascular smooth muscle. Beaudoin-Legros and Meyer [7] have reported an increase in Ca binding by microsomes of rabbit aorta under the influence of dibutryl cyclic AMP. This result was obtained both in the presence and in the absence of ATP in the incubation medium. In microsomal vesicles of the rat aorta, Webb [8] found a stimulation of Ca binding by cyclic AMP. In microsomes of canine aorta, cyclic AMP increased membrane phosphorylation when exogenous protein kinase was present but had no effect on Ca binding [9].

In the present study, using membrane fractions of the rabbit aorta, we provide evidence that both cyclic AMP and cyclic GMP have an inhibitory action on Ca uptake and on membrane phosphorylation by endogenous protein kinase, and a stimulatory effect on both processes when exogenous protein kinase is added.

Materials and Methods

Preparation of subcellular fractions. Adult rabbits of either sex weighing 2.5—2.8 kg were killed by a blow on the neck. The aorta was quickly removed, opened longitudinally and freed from the adventitia. The dissection was carried out in the buffer (sucrose 250 mM/EDTA 1 mM/Tris · HCl 20 mM, pH 7.4) used for the subsequent homogenization. The subcellular fractions were isolated from the media as follows: the vascular tissue (1.5—2.0 g wet weight) was cut into small pieces and homogenized for 3 min in an all-glass Potter-Elvehjem

homogenizer. The homogenate was centrifuged at 3 000 × *g* for 10 min and the supernatant was layered on a 30 ml 15—45% (w/v) continuous sucrose gradient. The gradient was centrifuged at 81 000 × *g* (g_{max}) for 90 min in a Beckman SW27 rotor. The fractions were collected by puncturing the centrifuge tube. After dilution to a sucrose concentration of 250 mM, the fractions were centrifuged at 100 000 × *g* for 1 h. The final pellets were resuspended in $MgCl_2$ 1 mM Tris · HCl 1 mM, pH 7.4, and used within 3 h. For measurement of phosphorylation $MgCl_2$ was omitted from the suspension medium.

Enzymatic assays. Assays for $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) were performed in 1 ml medium containing KCl 100 mM/NaCl 10 mM/$MgCl_2$ 5 mM/ATP 5 mM/Tris · HCl 10 mM, pH 7.4. 5'-Nucleotidase (EC 3.1.3.5.) was determined by the method of Song and Bodansky [10] and glucose-6-phosphatase (EC 3.1.3.9.) by the method of Solyom and Trams [11]. The inorganic phosphate liberated from substrates was measured by the method of Rockstein and Herron [12]. NADH-oxidase (EC 1.6.4.3.) was assayed as described by Ernster et al. [13] and cytochrome oxidase (EC 1.9.3.1) as described by Cooperstein and Lazarow [14]. All enzymatic assays were carried out at 37° C.

Assay of ^{45}Ca binding and uptake. ^{45}Ca uptake was measured in 1 ml solution containing KCl 70 mM/$MgCl_2$ 5 mM/ATP 5 mM/NaN_3 5 mM/histidine-HCl 20 mM, pH 6.8/$^{45}CaCl_2$ (2 μCi)/15—25 μg protein. Fixed concentrations of free Ca^{2+} lower than 10^{-5} M were obtained by addition of ethyleneglycol bis-(β-aminoethylether)-*N*,*N'*-tetraacetic acid (EGTA) buffer assuming a stability constant for Ca-EGTA of $5 \cdot 10^5\ M^{-1}$ [16]. For concentrations higher than 10^{-5} M, Ca^{2+} was calculated assuming stability constants for Mg · ATP and Ca · ATP of $6 \cdot 10^4\ M^{-1}$ and $2.5 \cdot 10^4\ M^{-1}$, respectively [17]. For measurements of oxalate-activated ^{45}Ca uptake, oxalate (5 mM) was added to the medium. The reaction mixture was incubated at 37° C for different periods of time. The incubation was terminated by filtration through a Millipore filter, which was washed with 15 ml ice-cold buffer. Blanks which contained all constituents except proteins were filtered simultaneously. The filters were then dissolved in 3 ml aethoxy-aethanol, to which 10 ml scintillator were added and counted in a liquid scintillation counter. The amount of ^{45}Ca uptake was calculated by correcting for the blank values and expressed in nmol ^{45}Ca/mg protein.

Assay of membrane phosphorylation. Phosphorylation of the membrane fractions was measured at 37° C in 0.4 ml solution containing Tris · HCl 50 mM, pH 7.5/$MgCl_2$ 10 mM/total ATP 30 μM/[γ-^{32}P]-ATP (50—70 μCi per sample)/15—25 μg protein. After 2 min preincubation, during which cyclic AMP, cyclic GMP and protein kinase were present, the reaction was started by the addition of ATP. Blanks containing 5 mM EDTA and 5 mM EGTA but no Mg^{2+} were run simultaneously. The reaction was stopped after 5 min by addition of 1 ml cold trichloroacetic acid, 7.5%. After centrifugation at 2500 × *g* for 15 min, the pellets were washed 3 times with the same solution and solubilized in 1 ml Soluene 350. Hydroxylamine sensitivity was tested by adding 1 ml 0.8 M hydroxylamine-HCl in 0.2 M acetate buffer (pH 4.0) for 30 min at room temperature after the 2500 × *g* centrifugation. 10 ml scintillator were then added and radioactivity was measured in a liquid scintillation counter. The amount of phosphorylation was calculated by correcting for the blank values and expressed in pmol ^{32}P/mg protein.

Other methods. Lanthanum-140 was tentatively used as marker for plasma membranes. Immediately after removal the aortae were incubated in 50 ml of a Ca-free solution (NaCl 148 mM/KCl 3.7 mM/$MgSO_4$ 0.81 mM/Tris · HCl 5 mM, pH 7.4) containing 0.15 mM $^{140}LaCl_3$ (20 μCi/ml) for 30 min at room temperature. During the incubation, the arteries were aerated with pure oxygen. The arteries were then washed 3 times for 5 min in 60 ml of the same solution but without La at 4° C in order to remove unbound La. Fractionation was then carried out as described. 0.1 ml of the resuspended final pellets were added to 0.5 ml Soluene 350 and 10 ml scintillator and counted in a liquid scintillation counter. In another type of experiment, fractions of the density gradient obtained by the normal procedure were incubated for 20 min in 1 mM $^{140}LaCl_3$, filtered on Millipore filters and counted for ^{140}La.

Protein was measured by the method of Lowry et al. [15].

For electron microscopy, pellets were fixed in 3% glutaraldehyde buffer (pH 7.2) and embedded in Epon after osmication and dehydration. Ultra-thin sections were stained with lead citrate and uranyl acetate.

Results

Isolation and characterization of membrane fractions. In agreement with other reports [18,19], no ($Na^+ + K^+$)-ATPase and only extremely low glucose-6-phosphatase activities were found in subcellular fractions of rabbit aorta. Therefore, 5′-nucleotidase was used as marker enzyme for plasma membranes and NADH-oxidase and oxalate-activated ^{45}Ca uptake as markers for endoplasmic reticulum. After fractionation of the homogenate in a continuous sucrose gradient, both 5′-nucleotidase and NADH-oxidase activities were found in the upper half of the gradient. For this reason, after discarding the first 18 ml of the lower part of the 30 ml gradient, 13 fractions of 1 ml each were collected, numbered 13 (heaviest fraction) to 1 (lightest fraction) and analysed for these two enzymes. The activity of 5′-nucleotidase peaked in the fractions 9 and 10 (Fig. 1) and was about 4—6 times higher there than in the supernatant of the first $3000 \times g$ centrifugation. Since lanthanum does not penetrate into the smooth muscle cells of rabbit arteries (Thorens, S. and Haeusler, G., manuscript in preparation) and since its efflux from this tissue is very slow (Thorens, S., unpublished), ^{140}La was tentatively used as a further marker for plasma membranes. After incubation of the aortae for 30 min in 0.15 mM ^{140}La prior to fractionation, the distribution of the radioactivity in the various fractions paralleled that of 5′-nucleotidase activity (Fig. 1). This characteristic distribution was not due to redistribution of the marker during the extraction process. Indeed, when the separated fractions instead of the intact arteries were incubated in ^{140}La, no preferential binding of the marker to any of the fractions was found (not shown). ^{140}La distribution as reported in Fig. 1 might, thus, well represent tight binding of the ion to negative sites of the plasma membrane. Since they corresponded to the bulk of both 5′-nucleotidase activity and ^{140}La binding, fractions 9 and 10 were considered as enriched in plasma membranes. They were pooled and termed fraction P. NADH-oxidase showed a peak of activity in fraction 3. However, NADH-oxidase activity was still very high in the heavier fractions. ^{45}Ca uptake in the presence of oxalate also

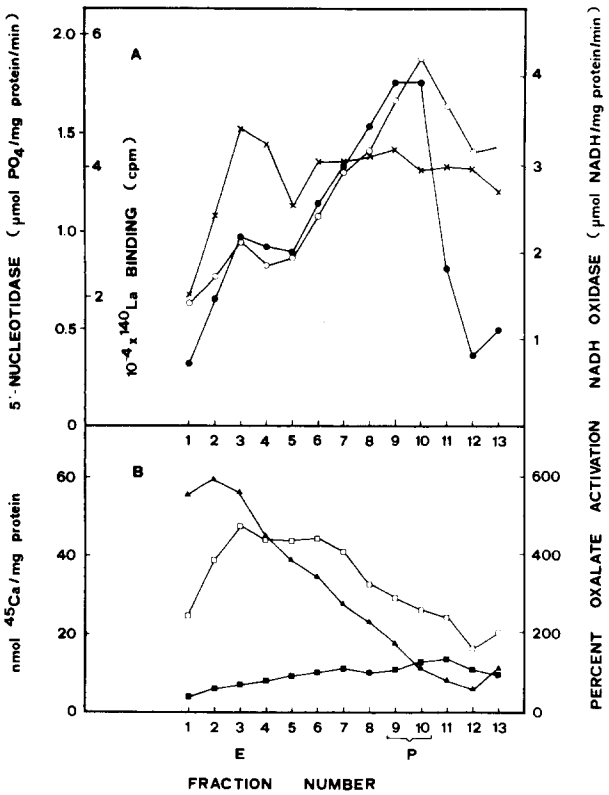

Fig. 1. Characterization of rabbit aorta subcellular fractions obtained by density gradient centrifugation. Activities of 5'-nucleotidase (•) and NADH-oxidase (X), ^{140}La binding (○) (A) as well as ^{45}Ca uptake in the absence (■) and in the presence (□) of oxalate 5 mM (B) were measured as described under Materials and Methods. ^{45}Ca uptake was measured after 10 min incubation at a Ca^{2+} concentration of 10^{-5} M. Increase in ^{45}Ca uptake by oxalate in percent (▲) was calculated from the corresponding values for ^{45}Ca uptake given on the same figure. Each point represents the mean value of 4—6 experiments. E and P refer to the fractions which were selected for further investigations.

showed a peak in fraction 3 (Fig. 1B). Moreover, oxalate-activation of ^{45}Ca uptake as expressed in percent was 4—5 times higher in the light fractions when compared with the heavy fractions. Fraction 3 was thus considered as enriched in endoplasmic reticulum and termed fraction E.

In electron micrographs, both the E and P fractions appeared as vesicular structures enclosed by smooth membranes. No intact mitochondria were found. The absence of any major mitochondrial contamination was confirmed by the measurement of cytochrome oxidase activity. While the highest activity of this mitochondrial enzyme was recovered in the density range between 40 and 45% sucrose, 16 and 10 times lower activities were found in fractions E and P, respectively. In order to prevent any possible interference by this small mitochondrial contamination, NaN_3 5 mM (as mentioned under Materials and Methods) was added to the incubation media when ^{45}Ca uptake was measured.

When the influence of Ca^{2+} concentration on ^{45}Ca uptake was measured in fractions E and P in the absence of oxalate, a higher K_m for ^{45}Ca uptake was found in fraction P ($K_m = 10^5$ M^{-1}) when compared with fraction E (K_m =

$2.9 \cdot 10^4$ M^{-1}). Very little ^{45}Ca uptake was observed in the absence of ATP. In both fractions, ^{45}Ca uptake with or without ATP in the medium showed a rapid phase lasting for about 1 min. In the presence of ATP, this rapid process was followed by a slow phase during which a continuous increase in ^{45}Ca uptake was observed. When oxalate was added, this increase proceeded linearly with time during at least 20 min and, therefore, changes in ^{45}Ca uptake measured after 10 or 20 min incubation reflect changes in the rate of ^{45}Ca uptake.

Oxalate activation of ^{45}Ca uptake as found in fraction P may reflect contamination of this fraction by endoplasmic reticulum (Fig. 1B). Alternatively, plasma membrane vesicles from smooth muscle, like those from skeletal [20] and cardiac muscle [21—23], might transport Ca in an oxalate-activated manner. For this reason, further studies on oxalate-activated Ca uptake were carried out in both E and P fractions.

Effects of cyclic AMP and cyclic GMP on Ca uptake. In both the E and P fractions no effects of cyclic AMP and cyclic GMP on ^{45}Ca uptake in the absence of oxalate were found, independent of the duration of the incubation period in ^{45}Ca or concentration of Ca^{2+} in the medium.

In both fractions, cyclic AMP slightly inhibited oxalate-activated ^{45}Ca uptake as measured after 20 min incubation in the presence of 10^{-5} M Ca^{2+} (Fig. 2, A and B). Cyclic AMP had no effect on oxalate-activated ^{45}Ca uptake at lower Ca^{2+} concentration. The effect of cyclic GMP on oxalate-activated ^{45}Ca uptake was similar to that of cyclic AMP; however, an inhibition was also detected with 10^{-7} M Ca^{2+} (Fig. 2, C and D). In fraction P, under the influence of cyclic GMP, slightly more inhibition was found at 10^{-5} M Ca^{2+} than 10^{-7} M Ca^{2+}. Shortening the periods of incubation as well as 10 min preincubation in cyclic AMP or cyclic GMP prior to addition of Ca gave identical results. Lower cyclic AMP or cyclic GMP concentrations (lowest concentration tested, 10^{-10} M) were inactive. AMP and GMP did not mimic the action of cyclic AMP and cyclic GMP.

It has been reported that in some preparations of heart sarcoplasmic reticulum, cyclic AMP activation of Ca uptake can only be demonstrated when exogenous protein kinase is added [6]. For this reason the effects of cyclic AMP and cyclic GMP were tested in the presence of beef heart protein kinase. In the presence of oxalate, protein kinase by itself stimulated the rate of ^{45}Ca uptake in both fractions (Table I), however, more stimulation was observed in fraction P than E. Stimulation of Ca uptake by exogenous protein kinase has been reported in heart muscle for both sarcoplasmic reticulum [6] and sarcolemma [24]. In fraction P, cyclic AMP, 10^{-6} M, produced statistically significant stimulations of the rate of ^{45}Ca uptake at two different protein kinase concentrations (Table I). Cyclic GMP, 10^{-6} M, had a similar influence on the rate of ^{45}Ca uptake but less marked. In fraction E, cyclic AMP stimulated the rate of ^{45}Ca uptake in the presence of 10 and 50 μg protein kinase per ml but these effects were not statistically significant. In the same fraction, cyclic GMP caused some stimulation of the rate of ^{45}Ca uptake only at the lower concentration of protein kinase.

Effects of cyclic AMP and cyclic GMP on phosphorylation. After 5 min incubation in [γ-^{32}P]ATP, both fractions showed Mg-dependent, hydroxylamine-insensitive, phosphorylation (Table II) at a rate which is comparable to

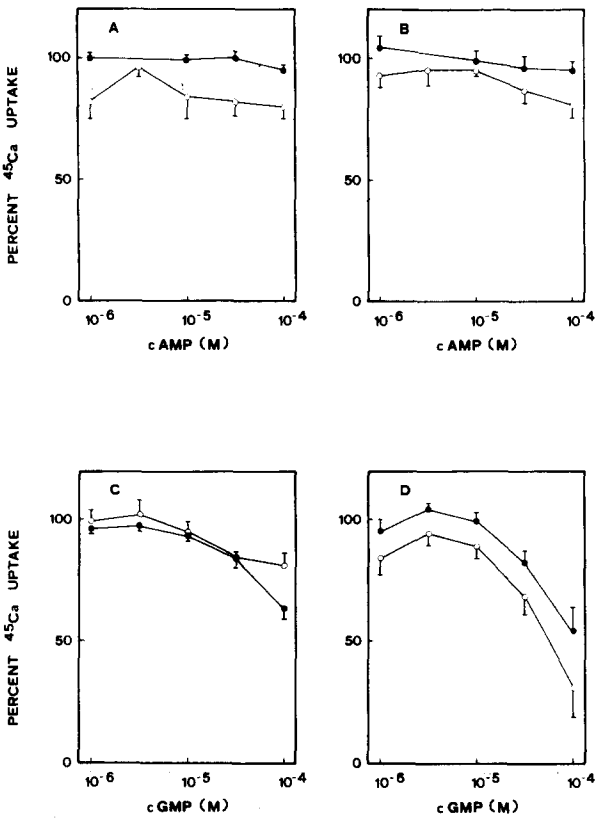

Fig. 2. Effects of cyclic AMP (cAMP) and cyclic GMP (cGMP) on the rate of ^{45}Ca uptake in aortic membrane fractions. ^{45}Ca uptake was measured after 20 min incubation in the presence of oxalate 5 mM at a Ca^{2+} concentration of either 10^{-7} (●) or 10^{-5} M (○) as described under Materials and Methods. A and C: fraction enriched in endoplasmic reticulum (fraction E). B and D: fraction enriched in plasma membranes (fraction P). Shown are the mean values ± S.E. of 6 experiments.

TABLE I

EFFECTS OF PROTEIN KINASE, CYCLIC AMP AND CYCLIC GMP ON OXALATE-ACTIVATED ^{45}Ca UPTAKE BY AORTIC MEMBRANE FRACTIONS

^{45}Ca uptake was measured after 10 min incubation as described under Materials and Methods. E, fraction enriched in endoplasmic reticulum; P, fraction enriched in plasma membranes. The numbers represent averages ± S.E. of n preparations.

	^{45}Ca uptake (%)	
	Fraction E	Fraction P
Control	100 ± 6 (n = 11)	100 ± 5 (n = 11)
Protein kinase 10 μg/ml	108 ± 6 (n = 10)	117 ± 3 [b] (n = 11)
Protein kinase 10 μg/ml cyclic AMP 10^{-6} M	117 ± 9 (n = 8)	146 ± 10 [c] (n = 8)
Protein kinase 10 μg/ml cyclic GMP 10^{-6} M	120 ± 7 [a] (n = 8)	122 ± 6 [a] (n = 8)
Protein kinase 50 μg/ml	119 ± 8 (n = 8)	142 ± 10 [b] (n = 8)
Protein kinase 50 μg/ml cyclic AMP 10^{-6} M	131 ± 9 [a] (n = 8)	178 ± 12 [d] (n = 6)
Protein kinase 50 μg/ml cyclic GMP 10^{-6} M	117 ± 6 (n = 8)	149 ± 10 [b] (n = 6)

[a] Significantly different from control ($P < 0.05$).
[b] Significantly different from control ($P < 0.01$).
[c] Significantly different from protein kinase 10 μg/ml ($P < 0.05$).
[d] Significantly different from protein kinase 50 μg/ml ($P < 0.05$).

TABLE II

EFFECTS OF Ca AND Mn ON ENDOGENOUS PHOSPHORYLATION IN AORTIC MEMBRANE FRACTIONS

Phosphorylation was measured as described under Materials and Methods. The numbers represent averages ±S.E. of n preparations.

Assay conditions	Phosphorylation (pmol ^{32}P/mg protein per 5 min)	
	Fraction E	Fraction P
$MgCl_2$, 10^{-2} M	245 ± 14 (n = 29)	318 ± 27 (n = 28)
$MgCl_2$, 10^{-2} M $CaCl_2$, 10^{-5} M	224 ± 24 (n = 7)	372 ± 24 (n = 7)
$MgCl_2$, 10^{-2} M $CaCl_2$, 10^{-4} M	292 ± 33 (n = 7)	467 ± 40 (n = 7) [a]
$MnCl_2$, 10^{-2} M	334 ± 27 (n = 4) [a]	807 ± 46 (n = 4) [a]

[a] Significantly different from the value of the first line ($P < 0.01$—0.001).

that reported for heart sarcoplasmic reticulum [25—27]. This result points to the presence of endogenous protein kinase(s) in membranes of rabbit aorta.

In both fractions phosphorylation was increased by the replacement of Mg^{2+} by Mn^{2+} (Table II). In the presence of 10^{-4} M $CaCl_2$, Mg-dependent phosphorylation was stimulated in fraction P but remained virtually unchanged in fraction E. No phosphorylation was observed when [γ-^{32}P]GTP was used as substrate. Cyclic AMP produced an inhibition of phosphorylation in both membrane fractions (Fig. 3A). This inhibition occurred at very low cyclic AMP concentrations (10^{-8} M). In contrast, AMP produced an inhibition only at the highest concentration tested (10^{-4} M). Cyclic GMP also inhibited phosphorylation (Fig. 3B). However, in fraction E, an inhibition was produced only at concentrations higher than 10^{-6} M. GMP caused some inhibition in fraction E at concentrations higher than 10^{-6} M but no inhibition in fraction P. Similar effects of cyclic AMP and cyclic GMP were found under the following experimental conditions: replacement of Mg^{2+} by Mn^{2+}, addition of 10^{-5} or 10^{-4} M $CaCl_2$ in the presence of Mg, 10-fold reduction in ATP concentration, shorter incubation periods.

Similar to Ca uptake, cyclic AMP-induced activation of phosphorylation in some preparations of heart sarcoplasmic reticulum is found only in the presence of exogenous protein kinase [25]. Beef heart protein kinase caused a stronger stimulation of phosphorylation than of Ca uptake: while 50 μg protein kinase per ml stimulated the rate of ^{45}Ca uptake by 40% in fraction P and 20% in fraction E (Table I), it activated phosphorylation by 220% in fraction E and 190% in fraction P (Fig. 4). At low protein kinase concentrations, higher stimulations of phosphorylation were found in fraction P than in fraction E.

In order to investigate the influence of cyclic AMP and cyclic GMP on phosphorylation in the presence of exogenous protein kinase, the experiments depicted in Fig. 3 were repeated with 10 μg heart protein kinase per ml. Under these conditions, cyclic AMP caused a stimulation of phosphorylation in fraction E (Fig. 5A), a result similar to those reported for heart sarcoplasmic reticulum [25]. In fraction P, no stimulation of protein kinase-catalyzed phosphorylation was observed; however, the inhibition caused by cyclic AMP in the

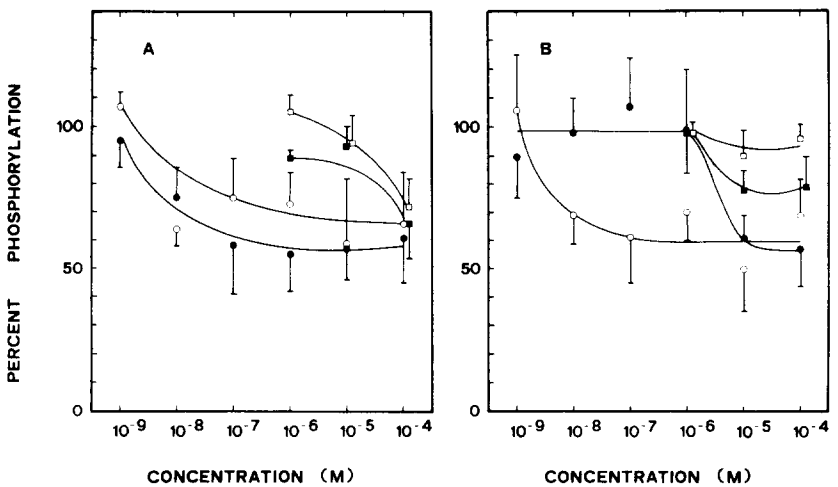

Fig. 3. Effects of nucleotides on phosphorylation in aortic membrane fractions. Phosphorylation was measured after 5 min incubation as described under Materials and Methods. A: comparison between the effects of cyclic AMP (circles) and of AMP (squares) in fraction E (filled symbols) and in fraction P (open symbols). B: comparison between the effects of cyclic GMP (circles) and GMP (squares) in fraction E (filled symbols) and in fraction P (open symbols). Shown are the mean values ± S.E. of 4—6 experiments.

absence of protein kinase was overcome. The effects of cyclic GMP on protein kinase-catalyzed phosphorylation were less marked than those of cyclic AMP particularly in fraction P where the inhibition caused by cyclic GMP in the absence of protein kinase was only partially reversed (Fig. 5B). Curves pre-

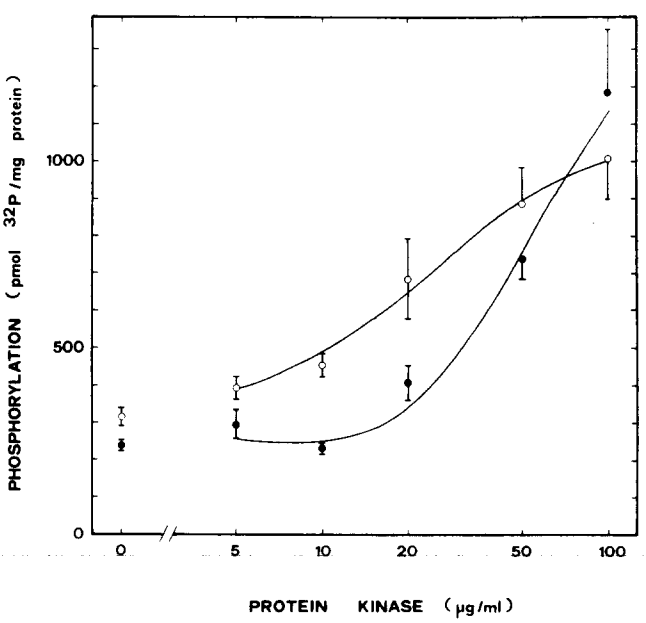

Fig. 4. Effects of beef heart protein kinase on phosphorylation in aortic membrane fractions. Phosphorylation was measured as described under Materials and Methods. The data have been corrected for phosphorylation attributable to the presence of added protein kinase. ●, fraction enriched in endoplasmic reticulum (fraction E); ○, fraction enriched in plasma membranes (fraction P). Shown are the mean values ± S.E. of at least 6 experiments.

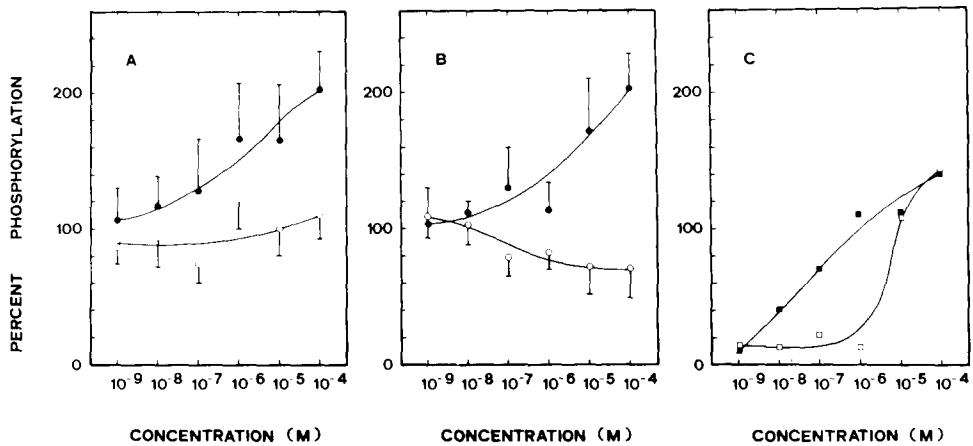

Fig. 5. Effects of cyclic AMP and cyclic GMP on phosphorylation in aortic membrane fractions in the presence of 10 μg beef heart protein kinase per ml. Phosphorylation was measured as described under Materials and Methods. A: effects of cyclic AMP in fraction E (•) and in fraction P (○). B: effects of cyclic GMP in fraction E (•) and in fraction P (○). C: differences between phosphorylation in the presence (data taken from Fig. 5, A and B) and in the absence of 10 μg beef heart protein kinase per ml (data taken from Fig. 3, A and B) in fraction E. ■, cyclic AMP; □, cyclic GMP. In A and B the mean values ± S.E. of at least 6 experiments are shown.

sented in Fig. 5C were constructed by substracting the data obtained in fraction E for the effects of cyclic AMP and cyclic GMP on phosphorylation in the absence of exogenous protein kinase (Fig. 3A) from those obtained in its presence (Fig. 5, A and B). This plot shows that the protein kinase-catalyzed phosphorylation is more sensitive to stimulation by cyclic AMP than by cyclic GMP.

Discussion

The membrane subfractions of rabbit aorta obtained in this study, particularly fraction E, show a high degree of oxalate-activated Ca uptake thus confirming reports on a potent Ca-accumulating system in both rabbit [28] and rat [8,29] aortae. The present results are, however, partly at variance with those of Allen [9] and Wei et el. [22] who reported high values for Ca binding but found no oxalate-activated Ca uptake in canine and rat aortae. The low affinity of aortic microsomes for Ca reported by Beaudoin-Legros and Meyer [7] and by Allen [9] is confirmed by the low K_m for Ca uptake found in the course of the present study. The high degree of oxalate-induced activation of Ca accumulation described here is in favour of intact vesicles in our membrane fractions. Moreover, the presence of reasonable high levels of endogenous phosphorylation makes these fractions a quite suitable material for studying the effects of cyclic nucleotides on the regulation of intracellular Ca.

With vascular tissue the separation of plasma membranes from endoplasmic reticulum is rendered difficult by the absence of a specific marker for the latter structure. The presence in a vesicular fraction of NADH-oxidase, the marker enzyme chosen for the endoplasmic reticulum in this study, is open to interpretation in different ways: it might result from (a) an enrichment in endoplasmic reticulum [30,31]; (b) the presence of outer membranes of mitochondria [32];

or (c) binding of the enzyme to plasma membranes subsequent to its detachment from the endoplasmic reticulum during the extraction process [13]. Interpretations b and c would explain the presence of high NADH-oxidase activities in fractions enriched in plasma membranes without implicating a contamination by endoplasmic reticulum. Due to the questionable distribution of NADH-oxidase among subcellular fractions, oxalate-activated Ca uptake is the only marker left for the localization of endoplasmic reticulum in smooth muscle. In accord with the results obtained in the aorta [29] and in mesenteric arteries [33] of the rat, oxalate-activated Ca uptake was principally recovered in a smooth vesicle fraction containing little activity in marker enzyme for plasma membranes.

Parallelism between ^{140}La binding and 5'-nucleotidase activity was found in their distribution among the fractions (Fig. 1). If this relationship is confirmed in other vascular tissues, La binding might represent a good non-enzymatic marker for plasma membranes of vascular smooth muscle cells.

The present study shows an inhibition of both oxalate-activated Ca uptake and phosphorylation by cyclic AMP and cyclic GMP in the absence of exogenous protein kinase and a stimulation of both processes, mainly by cyclic AMP, in its presence. As already mentioned under Results, alterations in the amount of ^{45}Ca taken up by the fractions reflect changes in the rate of ^{45}Ca uptake since ^{45}Ca uptake increased linearly with time. Inhibition of Ca uptake by cyclic AMP as described here has, to our knowledge, never been reported. Cyclic GMP has been shown to stimulate Ca release and to antagonize the stimulating effect of cyclic AMP on Ca binding in microsomes from rabbit colon [2]. At any rate, it is highly improbable that the inhibition of Ca uptake reported in this study is of physiological significance since it was only obtained at high concentrations (greater than 10^{-5} M) of both cyclic AMP and cyclic GMP.

Commercially available protein kinase has often been added to various membrane preparations in order to render their Ca accumulation sensitive to cyclic AMP. A common explanation for this action is an increase in phosphorylation resulting in a stimulation of Ca accumulation [6]. After this study was completed, Fitzpatrick and Szentivanyi [34] published a report describing an increase in Ca uptake by rabbit aorta microsomes in the presence of beef heart protein kinase and cyclic AMP. In the absence of exogenous protein kinase, cyclic AMP produced no activation of Ca uptake. Although these results have been confirmed in the present study, they have to be considered with caution. Before an action of cyclic AMP on Ca translocation via soluble protein kinase can be postulated for aortic smooth muscle, the effects on Ca uptake of cyclic AMP-dependent soluble protein kinase extracted from vascular tissues [37] must be clarified. Furthermore, the in vitro accessibility of soluble protein kinase to particulate substrates should be investigated. As to cyclic GMP, its action on Ca uptake in the presence of cyclic GMP-dependent protein kinase has to be established before firm conclusions on its interference with Ca movements can be drawn.

Evidence for the presence of Mg-dependent endogenous phosphorylation in aortic membrane fractions was found in this report. Since the phosphorylation is both acid stable and hydroxylamine insensitive, it is suggestive of phosphoester-type phosphorylation of the membranes by endogenous protein

kinase(s). Endogenous protein kinase activity was stimulated by Mn^{2+} in both fractions and by Ca^{2+} in fraction P (Table II). Stimulation by Mn^{2+} has been reported by Krause et al. [36] for soluble protein kinase. While phosphorylase kinase is activated by Ca^{2+} [38], soluble and particulate protein kinase are either inhibited [39,40] or unaffected [24,25,36] by this ion. If Ca^{2+}-induced stimulation of protein kinase from plasma membranes of vascular smooth muscle is confirmed, it would be an interesting feature of this enzyme and would differentiate it from protein kinase in membrane fractions of heart muscle. Inhibition of phosphorylation by cyclic AMP was observed in both fractions at very low cyclic AMP concentrations (10^{-8} M), whereas much higher concentrations of AMP (10^{-5}—10^{-4} M) were necessary for a comparable inhibition (Fig. 3A). This in favour of a specific action of cyclic AMP. Allen [9] has reported that cyclic AMP stimulated phosphorylation of histones by microsomes from canine aorta; the effects of cyclic AMP on autophosphorylation have, however, not been reported by this author. Inhibitory effects of cyclic AMP on phosphorylation have been described in membrane fractions from smooth muscle [41], in synaptic membranes [35] and in toad bladder [42]. This inhibition has generally been attributed to the stimulation of an endogenous protein phosphatase.

Inhibition of phosphorylation by low cyclic GMP concentrations was only observed in fraction P (Fig. 3B). In fraction E, both cyclic GMP and GMP caused inhibition at similar concentrations. Inhibition of phosphorylation by cyclic GMP has been reported by Sandoval and Cuatrecasas [43] using brain extracts and has been attributed to stimulation of protein phosphatase. In the case of aortic membrane fractions, further studies are necessary in order to clarify whether the inhibitory effect of cyclic AMP and cyclic GMP on phosphorylation results from an inhibition of endogenous protein kinase activity or from a stimulation of endogenous protein phosphatase.

In fraction E, in the presence of exogenous protein kinase, cyclic GMP apparently stimulated phosphorylation as much as cyclic AMP (Fig. 5, A and B). At first glance, this result was unexpected since the beef heart protein kinase used in this study is cyclic AMP dependent. However, if the effects of cyclic AMP and cyclic GMP on phosphorylation are considered as the sum of their effects on endogenous protein kinase (or endogenous phosphatase) and exogenous protein kinase, a reasonable explanation can be given. When the percent phosphorylation obtained in the absence of exogenous protein kinase is substracted from that obtained in its presence, the concentrations of cyclic AMP necessary for stimulation of phosphorylation ar much lower than the respective cyclic GMP concentrations (Fig. 5C).

In fraction P, in the presence of cyclic AMP or cyclic GMP, the addition of exogenous protein kinase had much less effect on phosphorylation than in fraction E (Fig. 5, A and B). The possibility that fraction P is a poor substrate for heart protein kinase must be discarded in the light of the result that at low concentrations of exogenous protein kinase more phosphorylation was observed in fraction P than E (Fig. 4). It remains, however, possible that the endogenous mechanism responsible for the inhibition of phosphorylation caused by cyclic AMP and cyclic GMP might be more efficient in this fraction than in fraction E. As discussed for Ca uptake, the physiological significance of the action of

cyclic AMP can only be determined when its final effect on phosphorylation of membranes via both the endogenous protein kinase-protein phosphatase system and the cytosolic protein kinase (from the same tissue) will be known. Determination of the effect of cyclic GMP on phosphorylation in the presence of soluble protein kinase will require the use of cyclic GMP-dependent protein kinase.

Although, in the absence of exogenous protein kinase, the effects of cyclic AMP and cyclic GMP on Ca uptake and phosphorylation were qualitatively similar, the fact that much higher concentrations of cyclic nucleotides were generally required to inhibit Ca uptake than to inhibit phosphorylation provides evidence against the existence of a coupling between these two processes in aortic membrane fractions. Although Ca uptake and phosphorylation were not measured under the same conditions, this result raises the possibility that in the intact tissue the modulation of endogenous phosphorylation by cyclic AMP and cyclic GMP might occur without an influence on Ca translocation. In the presence of exogenous protein kinase, 10^{-6} M cyclic AMP produced both an increase in phosphorylation and a stimulation of Ca uptake. This result suggests that in the case of phosphorylation by soluble kinase, Ca uptake and phosphorylation might be coupled. However, it should be noted that cyclic AMP stimulated Ca uptake more in fraction P than in fraction E, while the opposite was true for phosphorylation. Measurements of Ca uptake and phosphorylation under similar conditions would be necessary to estimate whether this lack of correlation results from the different experimental conditions or whether it reflects different properties of the two membrane fractions.

An antagonistic action between cyclic AMP and cyclic GMP has not been found either on Ca uptake or on phosphorylation. It has been proposed that cyclic AMP and cyclic GMP influence the tone of vascular smooth muscle in opposite ways [44]. If this were correct, our findings suggest that this antagonism is not the result of an interaction of the nucleotides with the Ca-accumulating system. The possible objection that cyclic GMP might inhibit Ca uptake in the presence of exogenous cyclic GMP-dependent protein kinase is of minor importance since cyclic GMP has been shown to stimulate phosphorylation by soluble cyclic GMP-dependent protein kinase from vascular tissues [37].

The results of the present study allow the conclusion that two subcellular fractions of smooth muscle from rabbit aorta, representing in all probability surface membranes and membranes of endoplasmic reticulum, possess endogenous protein kinase-protein phosphatase system(s) which mediate the cyclic AMP- or cyclic GMP-dependent phosphorylation of these membranes. The membrane phosphorylation regulated through this particulate protein kinase-protein phosphatase system seems to have no influence on Ca uptake by the two fractions and may serve other purposes. It is therefore suggested that cytosolic and not particulate protein kinase(s) mediate the effects of cyclic AMP or cyclic GMP on calcium translocation in vascular smooth muscle.

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