CONCERTED PHOSPHORYLATION OF ENDOGENOUS TRACHEAL SMOOTH MUSCLE MEMBRANE PROTEINS BY Ca^{2+} · CALMODULIN-, CYCLIC GMP- AND CYCLIC AMP-DEPENDENT PROTEIN KINASES

G. Kurt HOGABOOM†, Carol A. EMLER+, Fred R. BUTCHER+ and Jeffrey S. FEDAN†,*

†*Department of Pharmacology and Toxicology,* +*Department of Biochemistry, West Virginia University Medical Center* and *Physiology Section, Appalachian Laboratory for Occupational Safety and Health, NIOSH, CDC, DHHS, Morgantown, WV 26505, USA*

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1. Introduction

The precise control of the myoplasmic free $[Ca^{2+}]$ is an important determinant in the regulation of the contractile state of mammalian smooth muscle [1]. The control mechanism is thought to reside with membrane Ca^{2+}-transport ATPases which actively pump Ca^{2+} against a concentration gradient and maintain the intracellular $[Ca^{2+}]$ below 0.1 μM. The Ca^{2+}-transport ATPase has been measured in many smooth muscle types [2,3], purified [4], and its activity has been shown to be modulated by cyclic nucleotides [5,6] as well as by the Ca^{2+}-binding protein calmodulin [3].

The molecular mechanism by which calmodulin and cyclic nucleotides regulate smooth muscle Ca^{2+}-transport ATPase is unknown. The phosphorylation of the enzyme itself or the regulatory proteins may be associated with it. The latter mechanism has been developed for cardiac sarcoplasmic reticular Ca^{2+}-transport ATPase [7].

Here, we report the Ca^{2+} · calmodulin-, cyclic GMP- and the possible cyclic AMP-dependent phosphorylation of endogenous membrane proteins from tracheal smooth muscle. Two proteins, 130 000 and 80 000 M_r, appear to be phosphorylated by all 3 second messengers. The functional role of the concerted phosphorylation remains unknown but may be intimately involved in the regulation of smooth muscle membrane function including Ca^{2+} transport.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; TPCK, 1-tosylamide-2-phenylethyl chloromethyl ketone; EGTA, ethylene-glycol-(bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; PKI, cyclic AMP-dependent protein kinase inhibitor protein; CaM, calmodulin; TFP, trifluoperazine

* Address correspondence to J. S. F. at: Appalachian Laboratory for Occupational Safety and Health, WV 26505, USA

2. Methods

[γ-^{32}P] ATP was from New England Nuclear. Trifluoperazine was a generous gift of Smith, Kline and French.

Tracheal smooth muscle membranes were prepared as in [3] with the following modifications. The smooth muscle was homogenized in 8 vol. buffer A containing 250 mM sucrose, 10 mM Hepes (pH 7.0), 10 mM 2-mercaptoethanol, 0.5 mM PMSF, 0.05 mM TPCK, 0.5% ethanol, and 50 μg/ml heparin. The homogenate was filtered through 3 layers of cotton gauze. The filter residue was resuspended in 4 vol. buffer A, homogenized and filtered as above. Differential centrifugation of the combined filtrate was performed as follows: 1300 × *g*, 10 min; 10 000 × *g*, 10 min; 132 000 × *g*, 60 min. The 132 000 × *g* pellet used here was resuspended in buffer A lacking ethanol and heparin, placed on ice and used within 3 h.

Cyclic AMP- and cyclic GMP-dependent phosphorylation was assayed in 75 μl with 40 mM Hepes (pH 7.0), 10 mM KN_3, 15 mM EGTA, 10 mM Mg-acetate, 1 mM 3-isobutyl-1-methylxanthine, 100 μg protein, 5 μM MgATP, with or without cyclic AMP or cyclic GMP. Ca^{2+} · Calmodulin-dependent phosphorylation was assayed under identical conditions minus isobutylmethylxanthine and with or with-

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out Ca^{2+} and/or calmodulin. The $[Ca^{2+}]_{free}$ was determined with a Ca^{2+}–EGTA buffer system [3].

Following preincubation at 30°C for 4 min, reactions were begun with [γ-^{32}P]ATP (15 Ci/mmol), incubated for 1 min at 30°C, and terminated by adding 25 μl of a solution containing 280 mM Tris (pH 6.8), 8% SDS, 32% glycerol, 4% 2-mercaptoethanol and 0.008% bromophenol blue. Each sample was heated at 100°C for 2 min, covered, and allowed to stand overnight at room temperature.

Each sample was subjected to SDS–polyacrylamide gel electrophoresis as in [8] on 7–14% continuous polyacrylamide gradient gels. After autoradiography the appropriate protein bands were cut from the gels

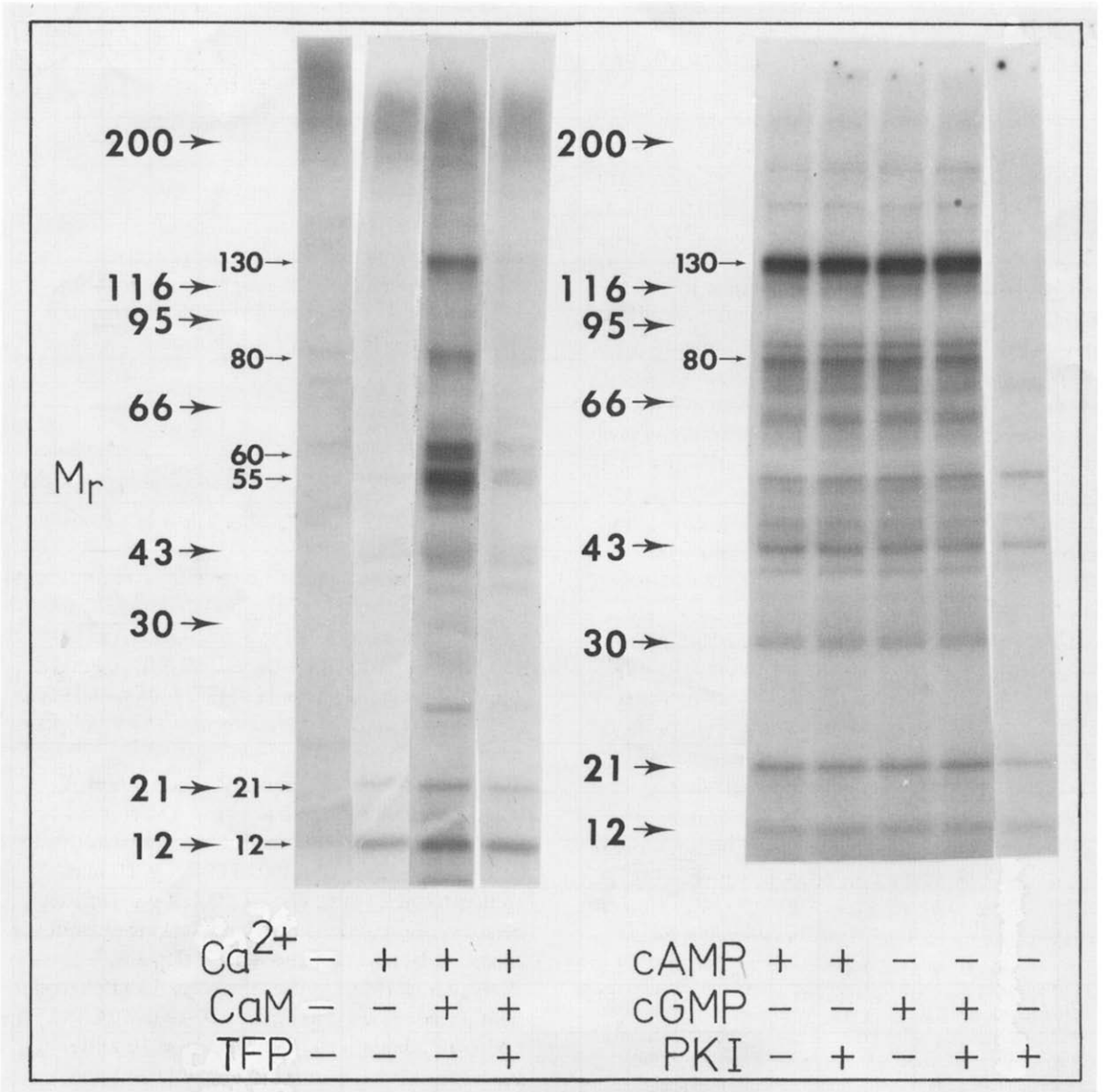

Fig.1. Autoradiograms illustrating phosphorylation of endogenous trachealis muscle membrane proteins. M_r standards ($\times 10^{-3}$) were: myosin (200), β-galactosidase (116), phosphorylase (95), bovine serum albumin (66), ovalbumin (43), catalase (30), trypsin inhibitor (21) and cytochrome *c* (12). The $[Ca^{2+}]_{free}$, [CaM], [cAMP] and [cGMP] were all 10 μM. The effect of 30 μM TFP was determined in the presence of 10 μM $Ca^{2+}{}_{free}$ and 0.3 μM CaM and the effect of 0.4 mg PKI/ml was assayed with 10 μM cAMP or cGMP. The autoradiograms are a representative example of $\geqslant$3 detn.

and ^{32}P-content assessed by scintillation spectrometry. Protein was determined as in [9].

3. Results and discussion

Preliminary experiments demonstrated the phosphorylation of membrane proteins to be linear for up to 90 s at 30°C in the presence of 5 μM ATP. Varying the [ATP] from 0.3–100 μM had no effect on the endogenous phosphorylation pattern.

Fig.1 illustrates the effect of various agents on membrane phosphorylation. Ca^{2+} and calmodulin together (calmodulin alone had no effect) stimulated the phosphorylation of several proteins, the most notable being 130 000, 80 000, 60 000, 55 000, 21 000 and 12 000 M_r. The majority of these proteins appear to be intrinsic to the membranes as washing with various salt concentrations (60–600 mM KCl) after the phosphorylation assay had no effect on the phosphorylation pattern. The only exception was the 21 000 M_r protein (unpublished). Trifluoperazine, an agent that binds to calmodulin and inhibits calmodulin-dependent processes [10], was found to specifically reverse the effect of Ca^{2+} and calmodulin.

Cyclic AMP and cyclic GMP also stimulated the incorporation of ^{32}P into 2 membrane proteins (130 000 and 80 000 M_r). These results are similar to the findings in the rat vas deferens and rabbit aorta [11]. The effect of cyclic AMP on membrane phosphorylation (fig.1) was not reversed by a concentration of PKI (0.4 mg/ml) that completely inhibits cyclic AMP-dependent phosphorylation in tracheal smooth muscle cytosol preparations (unpublished). The endogenous protein substrates also appear to be intrinsic to the membranes as washing with various salt concentrations (60–600 mM KCl) had no effect on the phosphorylation pattern (unpublished).

The effect of varying the $[Ca^{2+}]_{free}$ and [calmodulin] on Ca^{2+} · calmodulin-dependent phosphorylation was determined (fig.2). All proteins had similar $[Ca^{2+}]_{free}$- and [calmodulin]-dependencies with app. K_a for Ca^{2+} (3 μM) and calmodulin (0.1–0.5 μM) within known physiological concentration ranges [11,12].

The [cGMP]- and [cAMP]-dependencies of the 130 000 M_r and 80 000 M_r proteins were different with respect to their activation characteristics (fig.3). Cyclic GMP stimulated the incorporation of ^{32}P into the 130 000 M_r and 80 000 M_r proteins in a manner

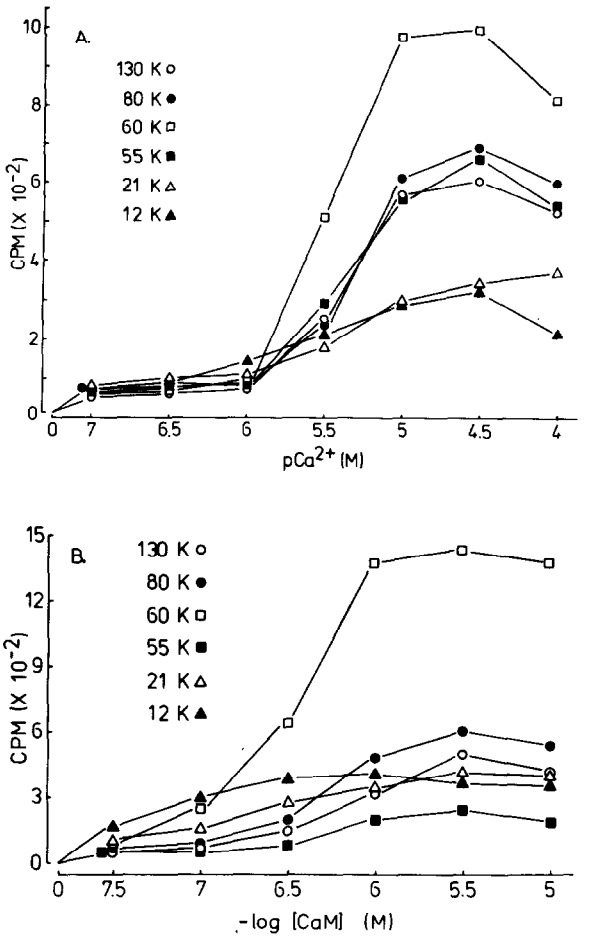

Fig.2. Ca^{2+} · CaM-dependent phosphorylation of endogenous tracheal smooth muscle membrane proteins as a function of $[Ca^{2+}]_{free}$ (A) or [CaM] (B). The effect of the $[Ca^{2+}]_{free}$ was determined in the presence of 10 μM CaM. The phosphorylation measured in the presence of 15 mM EGTA alone was subtracted from each value. The figures are representative examples of ⩾3 detn.

similar to the activation of several physiological processes by homogenous cyclic GMP-dependent protein kinase [13]. In contrast, cyclic AMP had an effect that is similar to the activation of cyclic GMP-dependent protein kinase by cyclic AMP [13]. These results and the finding that PKI had no effect on cyclic AMP, in this membrane preparation, may be due to the stimulation of an endogenous cyclic GMP-dependent protein kinase. Additional studies must be conducted to substantiate these results.

The physiological regulation of many cellular processes by second messengers such as Ca^{2+} and cyclic

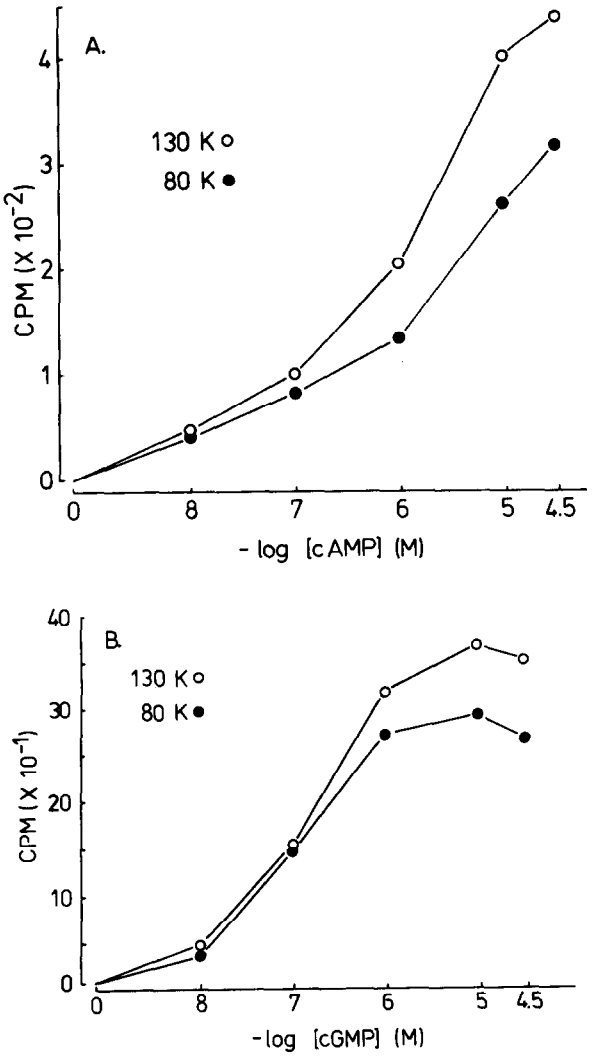

Fig.3. Phosphorylation of endogenous proteins from trachealis muscle as a function of the [cAMP] (A) or [cGMP] (B). The basal phosphorylation in the absence of the nucleotide was subtracted from each value. The figures represent an example of ⩾3 detn.

nucleotides is thought to occur through reversible phosphorylation mechanisms [14]. The specificity of a particular intracellular effector for a process may reside in the cellular location of endogenous substrate proteins with a tissue [15]. In addition, >1 intracellular mediator may regulate a system by a concerted phosphorylation as seen for brain protein I [16], phospholamban [17] and glycogen synthase [18].

These results suggest that Ca^{2+} · calmodulin and cyclic GMP, but not cyclic AMP, may regulate smooth muscle membrane function through the concerted phosphorylation of ⩾1 proteins. As calmodulin [3] and cyclic GMP [6] have been shown to stimulate smooth muscle microsomal Ca^{2+} transport, the phosphorylation of the Ca^{2+}-transport ATPase by these intracellular messengers may provide a common pathway by which the myoplasmic free [Ca^{2+}] may be precisely regulated in smooth muscle.

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