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Phosphorylation of Membrane Proteins by Protein Kinase in a Smooth Muscle Plasma Membrane Fraction (41426)

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Abstract. Properties of protein kinase and its phosphoprotein substrates associated with a plasma membrane (sarcolemmal) fraction isolated from rat uterine smooth muscle were investigated. The enzyme, a mixture of cAMP-dependent and -independent types, was active against exogenous substrates only in the presence of Triton X-100. In contrast, a few membrane proteins were actively phosphorylated in the absence of detergent. Protein kinase was resistant to trypsin digestion when associated with the plasma membrane but not when solubilized with Triton X-100. Membrane phosphoproteins, on the other hand, were sensitive to proteolysis in their native state. These results suggest the smooth muscle sarcolemmal protein kinase interacts extensively with the membrane lipid bilayer and its intrinsic substrates, though heterogeneous, share a common membrane orientation.

Membrane-associated protein kinases are widely distributed in nature but obscure in function (1). Proposed to regulate membrane permeability by phosphorylation of other membrane proteins which directly control diffusion or active transport (2), direct demonstration of this role has been presented only in the case of Ca^{+2} transport by heart sarcoplasmic reticulum (3, 4). One difficulty in relating the presence of protein kinase activity in membranes to function is that the variety of endogenous substrates may be large compared to the membrane processes they are proposed to regulate.

Even where function is uncertain, knowledge of the structural relationship between protein kinase and its substrates in the membrane might provide insight into the regulatory mechanisms involved. Characterization of the phosphorylation of proteins of purified plasma membrane isolated from uterine smooth muscle by intrinsic protein kinase suggests that even though these substrates were heterogeneous with respect to size, they seemed to have certain structural features in common and may therefore subserve a common function.

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Materials and Methods. *Fractionation of uterine smooth muscle cell membranes.* Uteri from between 6 and 15 Sprague–Dawley rats (200 g) were placed in ice-cold normal saline, trimmed of fat and connective tissue, and the endometrium removed by scraping with a microscope slide. Muscle strips were rinsed in saline and processed at 4° by Polytron homogenization in 10 vol of 0.25 *M* sucrose, 0.01 *M* Tris–HCl (pH 7.4), 0.01 *M* $MgCl_2$, and 0.0025 *M* dithiothreitol and the putative sarcolemmal fraction obtained by a combination of differential and density equilibrium centrifugation on discontinuous sucrose gradients according to our previously published procedure (5).

The membranes which banded at the 20/35% sucrose interface were concentrated as described and resuspended in the desired medium by hand homogenization. Protein concentration of all membrane suspensions was determined by the method of Lowry *et al.* (6).

Enzyme activity determination. 5'-Nucleotidase was determined as described in Krall and Korenman (5) except at substrate concentrations between 0.01 and 1.00 m*M*. Concentration-dependent hydrolysis was analyzed by reciprocal plot to obtain K_m (0.64 ± 0.11 μM) and V_{max}.

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TABLE I. COPURIFICATION OF PLASMA MEMBRANE PROTEINS WITH PUTATIVE SMOOTH MUSCLE SARCOLEMMA[a]

Activity	Filtered homogenate	Sarcolemmal fraction	n-Fold enrichment
5'-Nucleotidase[b]	550 ± 120	16,700 ± 2400	30×
Adenylate cyclase[c]	95 ± 5	1,300 ± 40	14×

[a] Mean ± SD of results from three separate preparations.
[b] nmole P_i/mg min.
[c] pmole cAMP/mg min in the presence of 100 μM NaF.

Protein kinase activity was quantified as the enzymatic transfer of radioactive phosphate from [γ-^{32}P]ATP to acid-insoluble exogenous substrate in the absence or presence of 0.1% Triton X-100, according to our previously published method (7). Results were corrected by subtraction for incorporation of radiophosphorous in the absence of the exogenous substrates.

Adenylate cyclase activity was determined as the enzymatic conversion of [α-^{32}P]ATP to [^{32}P]cAMP in 5 min at 37° as described in Krall and Korenman (8). Radioactive cAMP was subsequently purified by successive chromatography on columns of Dowex and aluminum oxide according to the procedure of Salomen *et al.* (9).

Analysis of membrane phosphoproteins by ^{32}P autophosphorylation and SDS–polyacrylamide gel electrophoresis. Membranes were incubated in the absence of detergent with [γ-^{32}P]ATP plus the desired additions at 2° for 2.5 min in a total volume of 0.125 ml of 0.1 M sodium phosphate buffer (pH 6.5), 0.025 M $MgCl_2$. Phosphorylation of endogenous protein was stopped by addition of 10% trichloroacetic acid and the insoluble precipitates electrophoretically analyzed on SDS–polyacrylamide slab gels (10) according to our previously published methods (8). Gels were stained with Coomassie brilliant blue, dried, and autoradiographed with X-ray film. Exposed and developed autoradiographs were scanned at 700 nm with a spectrophotometer equipped with a mechanical transport device. Molecular weights were estimated from the R_f of standards covering the range 212,000 to 12,600 which were coelectrophoresed with the membrane proteins.

Results. A uterine smooth muscle membrane fraction, more fully characterized elsewhere (5, 11), banded with an equilibrium density of 1.15 and was substantially

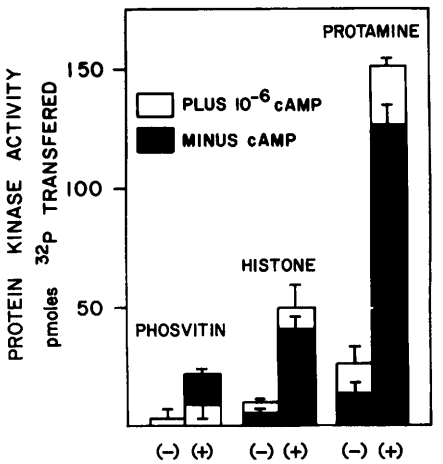

FIG. 1. Substrate specificity and detergent dependence of sarcolemmal protein kinase. Fifty micrograms of the purified membrane protein was incubated in a total volume of 0.125 ml of 0.1 M Na^+-phosphate buffer (pH 6.5) with 12.5 nmole ATP, 0.15 μCi [γ-^{32}P] ATP (29 Ci/mmole; New England Nuclear, Boston, Mass.), 6.25 μmole $MgCl_2$, and 225 μg of the indicated substrates in the absence (−) or presence (+) of 0.1% Triton X-100 and 10^{-6} M cAMP. Reactions were started by adding ATP and shifting the temperature from 2° to 30°. After 7 min, 1.0 ml of ice-cold 10% trichloroacetic acid (TCA) was added and the acid-insoluble precipitates were filtered onto glass fiber filters (Whatman GF/C) and washed with 3 ml of ice-cold 5% trichloroacetic acid. Dried filters were counted in a toluene-based scintillation fluid and the results corrected for membrane autophosphorylation in the absence of exogenous substrate. The results, the mean ± SD of three determinations of a single preparation, are representative of the properties of three separate preparations.

enriched in enzyme activities associated primarily with the plasma membrane (Table I). 5′-Nucleotidase activity was increased 30-fold by purification, while the somewhat lower enrichment in adenylate cyclase activity (determined in the presence of NaF) reflected the greater lability of that particular enzyme to the homogenization procedure employed. The purity of the fractions used in these studies were, therefore, equal to or greater than those characterized in (11).

The putative sarcolemmal fraction catalyzed the enzymatic transfer of phosphorous from ATP to a variety of exogenous phosphoprotein substrates (Fig. 1). Phosphorylation of two of these, histone and protamine, was slightly stimulated by cAMP, indicating the sarcolemma contained primarily cAMP-independent protein kinase or catalytic subunit of the cAMP-dependent enzyme but some holoenzyme, as well. Enzyme activity was stimulated up to 10-fold by the addition of low concentrations of the nonionic detergent Triton X-100.

Protein kinase associated with the membranes was insensitive to trypsin digestion (Table II). If the membranes were treated with Triton X-100 prior to incubation with trypsin, however, associated protein kinase was rendered sensitive to proteolysis. Soluble protein kinase activity present in the cytosol, previously shown to be the origin of an appreciable amount of the membrane enzyme in the uterine smooth muscle cell (7), was also trypsin sensitive (Table II).

When the plasma membrane fraction was incubated with [γ-^{32}P]ATP under autophosphorylating conditions, several membrane phosphoproteins were resolved by SDS–polyacrylamide gel electrophoresis and autoradiography (Fig. 2). Plasma membrane protein kinase substrates were present only at low concentrations, since no radioactive bands coincided with stained bands. Membrane autophosphorylation in the presence of cAMP resulted in a substantial reduction of labeling of a 64,500-molecular-weight peptide, but no increases in phosphorylation. Cyclic GMP increased labeling of a minor 174,000-molecular-weight band, and caused a small but reproducible decrease in labeling of the 64,500-molecular-weight species. When the membranes were incubated with trypsin, no plasma membrane protein phosphorylation was detected (Fig. 3). Trypsin sensitivity of

TABLE II. TRYPSIN SENSITIVITY OF PLASMA MEMBRANE COMPARED TO SOLUBLE PROTEIN KINASE[a]

Protein kinase source	(A) Inhibitor[b]		(B) Trypsin + inhibitor[b]		(C) Trypsin, no inhibitor[b]
	+ 1×10^{-6} *M* cAMP	No cAMP	+ 1×10^{-6} *M* cAMP	No cAMP	+ 1×10^{-6} *M* cAMP
I. Plasma membrane	96 ± 4	82 ± 10	128 ± 12	88 ± 14	0 ± 0
II. Triton-treated plasma membrane	88 ± 16	—	6 ± 5	—	0 ± 0
III. Cytosol	94 ± 17	66 ± 33	36 ± 9	4 ± 10	0 ± 0

[a] Membranes were suspended in 1.5 ml of 0.03 *M* histidine buffer (pH 7.5), 0.01 *M* $CaCl_2$ and divided into 0.5-ml aliquots (A, B, and C). 12.5 μg trypsin was added to B and C and all three aliquots were incubated at 30°. After 15 min 1000 units of the potent trypsin inhibitor Trasylol (FBA Pharmaceuticals) was added to A and B and incubation of all the aliquots continued for 5 min. The tubes were chilled on ice and 0.05-ml aliquots withdrawn for determination of protein kinase activity in the presence of 0.1% Triton X-100 according to the procedure in Fig. 1, using protamine as substrate. In experiment II (Triton-treated membranes), Triton X-100 was added before trypsin. Uterine smooth muscle cell post-100,000*g* supernatant was the source of the soluble enzyme (III, cytosol). Rat myometria were homogenized according to the procedure in Fig. 1 but in 10 vol of 0.03 *M* histidine buffer with 0.01 *M* $CaCl_2$. The homogenate was centrifuged at 100,000*g* for 60 min and 0.5-ml aliquots trypsin-treated and assayed for protein kinase activity as above but in the absence of Triton X-100. Results are the mean ± SD of triplicate determinations.

[b] Protein kinase activity in pmole ^{32}P transferred to protamine.

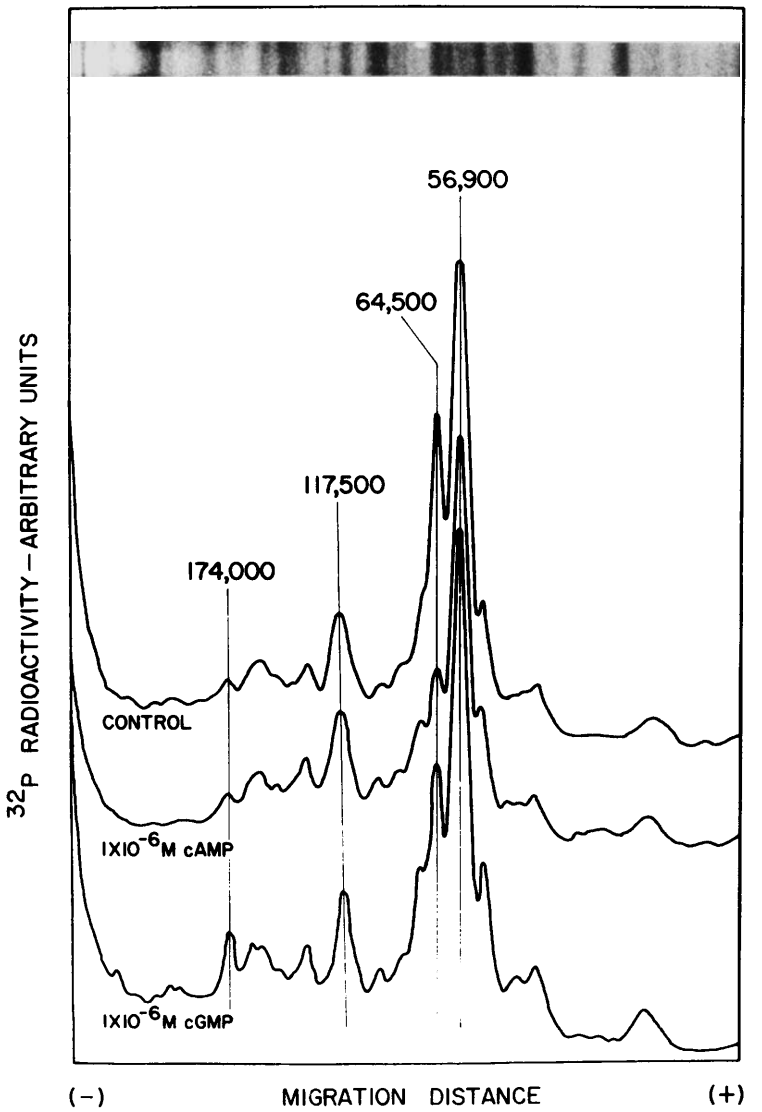

FIG. 2. Analysis of smooth muscle cell plasma membrane protein phosphorylation by intrinsic protein kinase. Membranes were incubated with the indicated additions prior to solubilization and electrophoretic analysis on 8.75% SDS–polyacrylamide slab gels as under Materials and Methods. Photograph of Coomassie brilliant blue-stained gel is at the top.

the endogenous substrates rather than enzymatic dephosphorylation was suggested since the same results were obtained whether the membranes were treated with trypsin before or following autophosphorylation. Trypsin resistance was a property shared by most of the smooth muscle cell plasma membrane proteins (5), so the trypsin-sensitive membrane substrates of intrinsic protein kinase were unique in that respect.

Discussion. Equilibrium centrifugation of smooth muscle microsomes on discontinuous sucrose gradients yielded three equilibrium density fractions. Properties of these have been characterized previously:

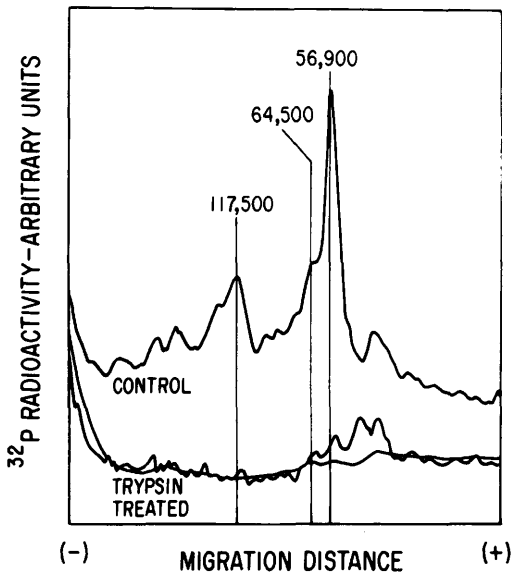

FIG. 3. Trypsin sensitivity of smooth muscle cell plasma membrane phosphoproteins. Membranes were either incubated with trypsin in the absence of Triton X-100 according to the procedure in Table II, then phosphorylated and electrophoretically analyzed as in the legend of Fig. 2 following the addition of trypsin inhibitor; or phosphorylated first, then treated with trypsin. Membranes were incubated under the same conditions but in the absence of trypsin and phosphorylated in the presence of 10^{-6} *M* cAMP as a control.

the lightest (d = 1.15) was high in plasma membrane marker activity, free of nuclear and mitochondrial contamination, but may contain small amounts of sarcoplasmic reticulum although this was judged unlikely based on the uniform resistance of proteins to salt extraction and trypsin digestion (5). Substantial increases of β-adrenergic catecholamine and PGE receptors (11) and in adenylate cyclase activity, proteins associated principally with the plasma membrane of hormone-sensitive target cells (12–14), as well as in 5'-nucleotidase activity suggested appreciable purification of the smooth muscle cell sarcolemma had been achieved.

In addition, the fraction contained a protein phosphorylating enzyme suggesting that some, if not much, of the protein kinase activity previously reported as associated with uterine smooth muscle cell membranes was sarcolemmal in origin (7). The membrane-associated enzyme had several properties associated with "integral" membrane proteins, including resistance to extraction by high- and low-ionic strength buffers and trypsin resistance, suggesting the enzyme interacts with the lipid bilayers. From the properties described above, there seem to be only a limited number of ways that protein kinase and its phosphoprotein substrates may be organized within the plasma membrane. Sarcolemmal protein kinase is most likely a mixture of cAMP-dependent and -independent varieties as reflected by the low but significant stimulation of enzyme activity by the cyclic nucleotide (Fig. 1). The catalytic subunits of both types of activity are therefore protected from proteolysis, perhaps by interaction with the lipid bilayer. Hydrophobic interaction is further suggested by the large dependence of enzyme activity toward exogenous substrates on the presence of a nonionic detergent, a property not shared by the endogenous membrane substrates.

Of the numerous sarcolemmal proteins, only a few minor species were protein kinase substrates and at least part of these phosphoprotein molecules were probably also embedded in the bilayer. The phosphorylation of these hydrophobic portions is consistent with both the apparent hydrophobic nature of the trypsin-resistant uterine smooth muscle cell protein kinase and with the characterization of the phosphorylated sites of substrates for protein kinase in other tissues as hydrophobic in nature (15, 16). The trypsin sensitivity of the sarcolemmal substrates indicates that part of the phosphoprotein molecule also protrudes from the bilayer, however.

There have been few investigations of the organization of protein kinase and its substrates within membranes. Characterization of protein kinase activity associated with erythrocyte membranes indicated that enzyme was localized to the cytoplasmic side of the plasma membrane (17). This asymmetric distribution may include several different varieties of protein kinase associated with the erythrocyte membrane,

and phosphorylation of both integral membrane proteins and peripheral proteins such as spectrin probably occurs (18, 19). In the myometrial sarcolemma, on the other hand, phosphorylation seems to be restricted to proteins which interact with the lipid bilayer. Thus, the erythrocyte envelope and the myometrial sarcolemma are interesting contrasts in the structural association between membrane protein kinases and their substrates. Such differences may reflect quite different functional roles for membrane protein phosphorylation.

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