THE DEVELOPMENT OF cAMP-DEPENDENT PROTEIN PHOSPHORYLATION IN SKELETAL MUSCLE SARCOLEMMA

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1. Introduction

Protein phosphorylation in mammalian skeletal muscle is related to diverse activities including the control of: (i) glycogen and lipid metabolism; (ii) calcium transport by the sarcoplasmic reticulum; (iii) hormonal regulation of contractile tension and rate of relaxation [1]. Protein kinase enzymes located in the sarcolemmal membrane have been shown to catalyze the transfer of terminal phosphate from ATP to endogenous membrane polypeptides and exogenous substrates such as histones. In [2] protein kinase activity was examined in sarcolemma prepared from rat hind limb skeletal muscle and the phosphorylation of two membrane peptides of M_r 88 000 and 15 000 observed. Kinase activity was stimulated 40% by insulin (1 munit/ml), implicating sarcolemmal protein phosphorylation as a possible step in the enhancement of glucose transport and/or Ca^{2+} efflux by insulin. In [3] protein kinase activity of a M_r = 51 000 peptide in diaphragm muscle sarcolemma was identified. Similar to [2], activity was not stimulated by cAMP and the incorporated phosphate was alkalilabile.

In [4] the postnatal development of skeletal muscle sarcolemma was paralleled by a 6–7-fold increase in epinephrine-stimulated adenylate cyclase activity. Here we show that the increase in adenylate cyclase activity is accompanied by the appearance of cAMP-dependent protein kinase activity for histones and 2 membrane polypeptides of M_r 48 000 and 55 000.

2. Materials and methods

Newborn (0–1 week) and adult (8 week) New Zealand white rabbits were the source of lower limb skeletal muscle. An enriched fraction of sarcolemmal membrane was isolated as in [4]. Briefly, a muscle homogenate was prepared by Polytron disruption (setting 7.5, 15 s) in sucrose buffer (0.25 M sucrose, 0.1 mM Tris–HCl, 0.1 mM Na_2EDTA (pH 7.6)). The nuclear fraction from this homogenate was extracted sequentially with lithium bromide and KCl medium to yield a crude membrane fraction. Membranes were resolved by gradient centrifugation over 15–35% continuous sucrose gradient. Material distributing between the 18–23% zone of the gradient possessed the highest total and specific activity for $Na^+,K^+(Mg^{2+})$-ATPase, adenylate cyclase and β-adrenergic receptor and were used for the determination of protein kinase activity.

2.1. *Protein kinase determination*

Membrane material (50 μg) was incubated in 100 μl final vol. containing 50 mM Tris–HCl, 0.05 mM Tris–ATP, 10 mM $MgCl_2$, 1 mM Na_2EDTA (pH 7.5) with $1–5 \times 10^6$ cpm [γ-^{32}P]ATP. The reaction was terminated by addition of 100 μl 2% SDS, 1 mM dithiothreitol, Tris–HCl, 20% sucrose (pH 7.5). Solubilized membranes were analyzed by 1% SDS–7.5% polyacrylamide gel electrophoresis and stained with 0.25% Coomassie blue to reveal total membrane peptides [5]. Gels were sliced in ~0.5 mm slices, decolorized with hydrogen peroxide, and liquified by heating for quantitation of radioactive phosphate incorporation [6].

Protein kinase activity in the total muscle homogenate was measured in a reaction mixture (0.2 ml) consisting of 50 mM KH_2PO_4, 0.5 mM methylxanthine, 5 mM $MgCl_2$, 20 mM NaF, 1 mM Tris–ATP ($1–5 \times 10^6$ cpm [γ-^{32}P]ATP), 100 μg calf thymus histone IIA (pH 7.5) [7,8]. The reaction was initiated

by the addition of 100 μg homogenate protein and incubated at 30°C over a time span of 0–10 min. The reaction was terminated by addition of 1.0 ml ice-cold 5% trichloroacetic acid and the protein precipitate isolated by filtration (Millipore filters, type HA, 0.45 μm). The precipitate was washed twice with ice-cold 5% trichloroacetic acid and once with cold 95% ethanol. The filters were dried and radioactivity quantitated by liquid scintillation spectrophotometry. The incorporation of radioactive phosphate was linear with respect to time and protein concentration for both neonate and adult muscle total homogenates.

Protein determination were by the Lowry method, using bovine serum albumin as the standard [9].

[γ-^{32}P]ATP, 1000–3000 Ci/mmol was purchased from New England Nuclear. Rabbit muscle protein kinase inhibitor (Sigma Chemical Co.) inhibited 200 units protein kinase/mg protein (unit = transfer of 1 picomol phosphate . min^{-1} . mg^{-1}).

3. Results

Distinct differences in the pattern of phosphate incorporation into membrane peptides and histone were observed between neonatal and adult sarcolemma (fig.1 A,B). In the absence of 0.1 mM cAMP adult sarcolemma exhibited protein kinase activity for a membrane polypeptide of M_r 80 000 and exogenously-added histone. Cyclic AMP enhanced the phosphorylation of these peptides ~2–3-fold. Additionally, in the presence of cAMP two membrane peptides of M_r 48 000 and 55 000 were phosphorylated 5–6-fold greater than in the absence of cAMP. Neonatal sarcolemma did not have protein kinase activity for any of the native membrane polypeptides. The M_r 55 000 peptide substrate of adult sarcolemma was not present in neonatal membranes as judged from the Coomassie blue-staining profile of the SDS gel. Neonatal membrane demonstrated protein kinase activity for only exogenously added histone which was not significantly activated by cAMP.

The rate and extent of phosphate incorporation into membrane polypeptides and histones is shown in fig.2A,B. At 10 μg histone phosphorylation was linear in both adult and neonatal sarcolemma for the initial 2 min of the experiment. The initial rate of histone phosphorylation by adult sarcolemma (100 pmol phosphate . min^{-1} . mg^{-1}) was 6–7-fold greater than neonatal membranes (15 pmol phosphate . min^{-1} . mg^{-1}) indicating elevated levels of protein kinase enzyme activity and not limitation of protein substrate to explain the higher capacity of adult sarcolemma for protein phosphorylation. The most rapidly labelled membrane substrate was the M_r 80 000 polypeptide which reached maximum extent of phospho-

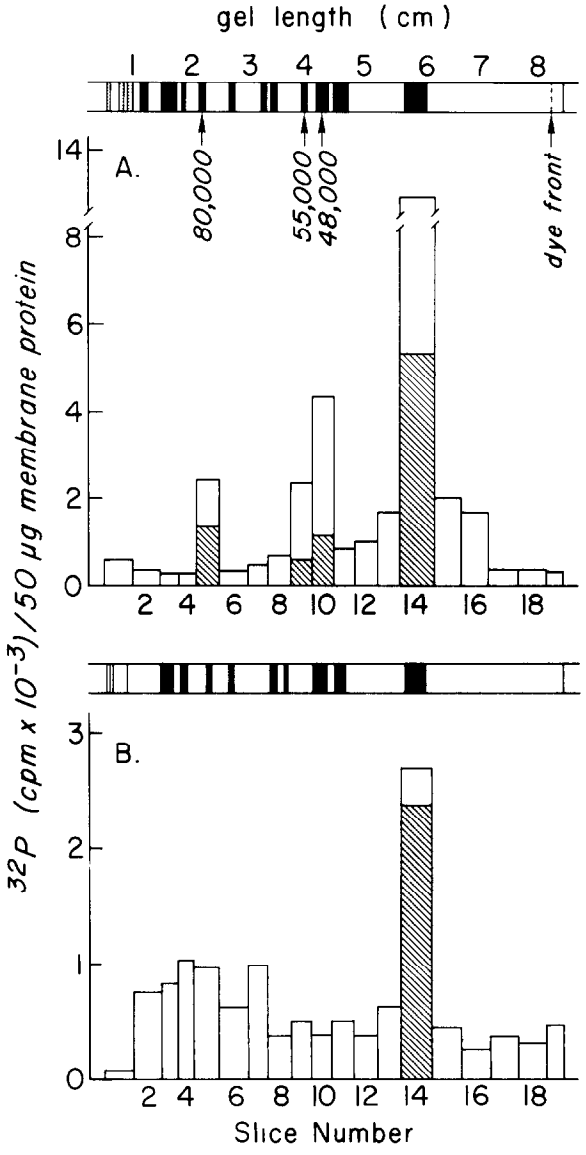

Fig.1. Polypeptide and phosphorylation pattern of sarcolemmal membranes. Adult (A) and neonatal (B) sarcolemma were incubated 1 min in the presence of either 10 μg histone (▨), or 10 μg histone and 0.1 mM cAMP (□). The Coomassie blue staining pattern corresponds with the slice number and amount of radioactive phosphate incorporated. For adult sarcolemma, slices 5, 9 and 10 correspond with endogenous membrane substrates of M_r 80 000, 55 000 and 48 000, respectively; slice 14 corresponds with histone.

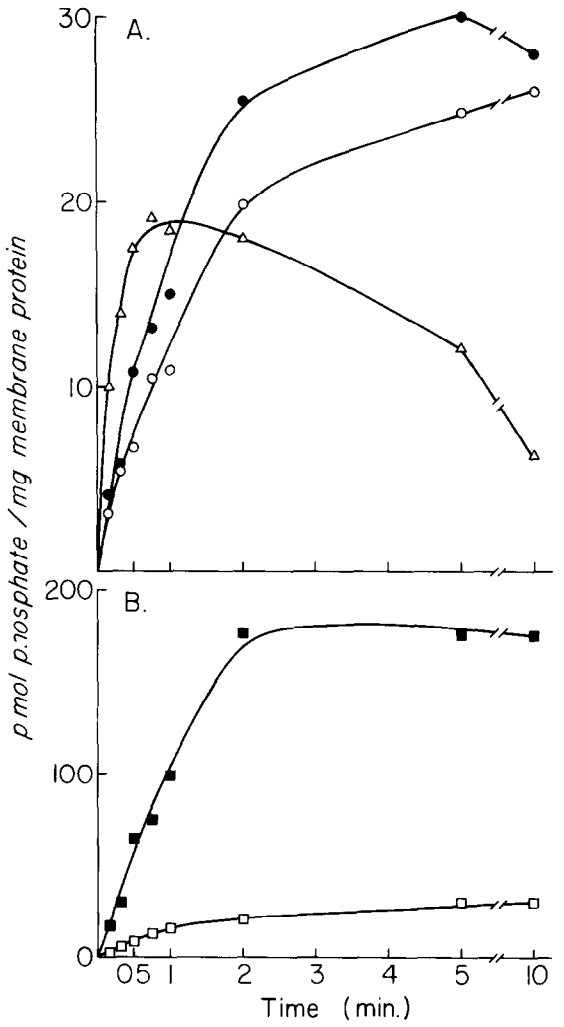

Fig.2. Kinetics of protein kinase activity. Sarcolemmal membranes were incubated in a reaction mixture containing 10 μg histone and 0.1 mM cAMP. At the indicated time intervals, membranes were solubilized and gel electrophoresis performed to determine incorporation of ^{32}P into individual peptides. Membrane protein phosphorylation (A), M_r 80 000 (△——△); M_r 55 000 (○——○) and M_r 48 000 (●——●). Histone phosphorylation (B), adult (■——■); neonate (□——□).

rylation by the initial 45 s of the reaction. Thereafter this peptide progressively lost radioactivity. The M_r 48 000 and 55 000 polypeptides exhibited similar initial rates of phosphorylation (20–22 pmol phosphate . min^{-1} . mg^{-1}).

Maximal stimulation of protein kinase activity was achieved with cAMP at 0.01 mM (table 1). In the presence of protein kinase inhibitor the cAMP-stimulated component of phosphate incorporation was inhibited 70–80% for both the endogenous membrane polypeptides and histones in adult sarcolemma. Histone phosphorylation in the absence of cAMP was not inhibited by protein kinase inhibitor, indicating that this phosphorylation reaction is not catalyzed by the free catalytic subunit of cAMP-dependent protein kinase. Histone phosphorylation in neonatal sarcolemma was not inhibited by protein kinase inhibitor in accord with the inability of cAMP to stimulate phosphorylation.

In agreement with work on developing rat skeletal

Table 1
Effect of protein kinase inhibitor (PKI) on sarcolemmal protein kinase activity

Condition	Substrate (pmol phosphate . 2 min^{-1} . mg^{-1})		
	80 000	48 000	Histone
– Cyclic nucleotides	10.6	2.4	42
0.01 mM cAMP	15.3	19.9	136
0.01 mM cAMP + PKI	11.8	5.4	62
– Cyclic nucleotides + PKI	10.2	2.8	45

Sarcolemmal membrane protein kinase determinations were performed as in section 2. For inhibition studies, membranes (50 μg protein) were preincubated for 5 min at 30°C in the presence of 25 μg protein kinase inhibitor protein

Table 2
Total protein kinase activity

	– cAMP	+ cAMP (0.01 mM)
Neonate		
Spec. act. (pmol . min^{-1} . mg^{-1})	52.5 ± 4.1	152.3 ± 15.2
Total act. (pmol . min^{-1} . g^{-1})	3412.5 ± 316	9899.5 ± 1037
Adult		
Spec. act.	35.6 ± 4.7	46.7 ± 9.4
Total act.	3738.0 ± 504	4903.5 ± 1106

Protein kinase activity employing 100 μg calf thymus IIA histone as the acceptor substrate was performed as in section 2. Radioactivity in the 0 time and/or minus histone reaction was subtracted from each time point to determine net radioactive phosphate incorporated. Total activity is expressed as pmol P_i incorporated/min for 1 g (wet wt) of neonatal and adult skeletal muscle. The calculation of total activity was based on the protein content of 1 g (wet wt) of skeletal muscle; these values were 65 mg protein/g and 115 mg/g for neonate and adult, respectively

muscle [10], total protein kinase activity and sensitivity to cAMP stimulation declined during development (table 2). Neonatal enzyme was stimulated 3-fold (52.5 *vs* 152.3 pmol phosphate . min^{-1} . mg^{-1}) by 0.01 mM cAMP whereas adult enzyme only showed a 1.3-fold (35.6 *vs* 46.7) stimulation. In the absence of cAMP both the specific and total activities for protein kinase were similar; total cAMP stimulated activity for neonatal muscle (9899 pmol . min^{-1} . g^{-1}) was increased 2-fold compared to adult (4903 pmol . min^{-1} . g^{-1}). Experiments performed on a 100 000 × *g* supernatant fraction of the total homogenate yielded identical results, indicating that the cytosolic and sarcolemmal membrane protein kinases undergo inverse changes with respect to cAMP dependency and enzyme activity during muscle development.

4. Discussion

The role of membrane protein phosphorylation in excitable tissues has variously been associated with microtubule function, neurotransmitter synthesis and postsynaptic actions of neurotransmitters. Dopaminergic synapses of the central nervous system in [11] responded to dopamine with increased adenylate cyclase activity and cAMP synthesis. Cyclic AMP in turn activates membrane-bound protein kinase to phosphorylate two membrane proteins termed protein I and protein II. It is believed that the cyclic phosphorylation–dephosphorylation of these synaptic membrane proteins is related to altered sodium and potassium permeability induced by dopamine. In [12] sodium and potassium efflux and the resting membrane potential of skeletal muscle were elevated by physiological levels of the catecholamines. The effect of epinephrine on sarcolemma electrical properties was mimicked by dibutyryl cAMP and blocked by the β-adrenergic receptor antagonist, propranolol, suggesting the function of a coupled β-adrenergic receptor–adenylate cyclase system in the control of sarcolemma ionic permeability. The development of skeletal muscle is accompanied by increased sodium and potassium permeability, Na^+,K^+(Mg^{2+})-ATPase activity and magnitude of the resting membrane potential [13,14]. In this and previous studies, developing sarcolemma acquired increased epinephrine adenylate cyclase activity, cAMP-stimulated protein kinase activity and endogenous membrane protein phosphorylation. The coordinate appearance of these sarcolemmal activities may be related to the expression of altered membrane electrical properties in developing skeletal muscle and control by the catecholamine hormones.

Acknowledgements

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