J. Membrane Biol. 49, 123–138 (1979)

Phosphorylation of Casein by Human Erythrocyte Membrane-Bound Protein Kinase: Competition of Casein with Endogenous Substrates

J.D. Vickers, J. Brierley, and M.P. Rathbone

MRC Group in Developmental Neurobiology, Department of Neurosciences, McMaster University Medical Centre, Hamilton, Ontario, Canada, L8S 4J9

Received 9 October 1978; revised 13 February 1979; revised again 27 April 1979

Summary. The possibility that spectrin and band-3 protein are phosphorylated by the same membrane-bound protein kinase was investigated by adding casein to unsealed erythrocyte ghosts and examining competition of the three proteins for phosphorylation. The extent of spectrin and band-3 protein phosphorylation was reduced by up to approximately 55%. This indicated that casein was competing with these endogenous substrates for phosphorylation and was most probably phosphorylated by the same protein kinase(s). Furthermore, the extent of inhibition of the phosphorylation of the two endogenous substrates was indistinguishable over the range of casein concentrations tested (0.1 to 5 mg/ml). This indicates that spectrin and band-3 protein may be phosphorylated by the same protein kinase. In contrast, casein was found to have no effect on the cAMP-dependent phosphorylation of band 4.5. This result indicates that casein only competes with the endogenous proteins phosphorylated by the cAMP-independent protein kinase(s).

The extent of reduction of endogenous substrate phosphorylation in the presence of casein was found to be constant over incubation periods of 1 to 15 min, indicating that this reduction was not due to consumption of ATP.

Since the spectrin and band-3 protein phosphorylations were specifically and identically reduced by casein and these reductions were not due to the ATP consumption or to a general alteration of the membrane, we conclude that the two substrates are likely phosphorylated by one kinase which also phosphorylates casein.

The study of membrane-bound enzymes is complicated by the requirement of such enzymes for the normal membrane environment for their physiological activity and specificity [6, 7, 15, 25]. Some of the erythrocyte membrane protein kinases when removed from the membrane together with the other membrane proteins lose their normal substrate specificities [8]. Thus, it is difficult to study particular protein kinases responsible for erythrocyte spectrin and band 3-protein phosphorylation after extraction from the membrane, and techniques to examine the interactions of the protein kinase(s) and substrates *in situ* are required.

The regulation of phosphorylation of spectrin and band-3 protein is of particular interest since the phosphorylation of these proteins is

0022-2631/79/0049-0123 $03.20

abnormal in patients with myotonic and Duchenne muscular dystrophy [1, 16, 17, 21–23]. This alteration of enzyme activity has been suggested to be secondary to a general alteration of plasma membranes in these diseases [1, 14, 19, 21–23]. We have found that changes of these two protein phosphorylations are highly correlated with each other both in erythrocytes from patients [21–23], and in erythrocytes with artificially altered lipid compositions [18, 24]. These results can be interpreted as resulting from either a general alteration in the membrane affecting two protein kinases comparably or from a specific effect upon a single enzyme species which phosphorylates both protein substrates. Avruch and Fairbanks [3, 8] initially suggested that there might be two cAMP-independent protein kinases on the basis of different ionic requirements for the phosphorylations. In a later study, however, parallel elution of the two protein kinase activities from the membrane led them to suggest that only one enzyme might be present [4]. The difference in ionic requirement observed might be due to specific ionic requirements of the two substrates required for optimal interaction with the membrane environment and the enzyme.

More recently Hosey and Tao [11] demonstrated that a cAMP-independent protein kinase, extracted from erythrocyte membrane phosphorylates both spectrin and band-3 protein when added to heat-inactivated membranes. Furthermore, both substrates were phosphorylated when GTP was used as the phosphate donor although at a lower efficiency (25%). Partial purification by $(NH_4)_2SO_4$ precipitation and gel filtration did not resolve this activity into multiple fractions (unlike the rabbit erythrocyte kinase) [12], indicating that one protein kinase is responsible for the phosphorylation of both substrates.

Avruch and Fairbanks [3] and Hosey and Tao [12] reported that casein is phosphorylated by a cAMP-independent protein kinase in spectrin-depleted erythrocyte vesicles and that the reaction has specific ionic requirements (stimulation by Na^+) similar to those of the spectrin kinase. A major difference, that is as yet unexplained, is that casein is not phosphorylated by spectrin kinase when GTP is used as a phosphate donor [11]. We found that casein was equally well phosphorylated by protein kinase(s) in erythrocyte membranes (ghosts) in which spectrin was still present. This permitted us to investigate the possibility that casein competes with spectrin and band-3 protein for phosphorylation and that spectrin and band-3 protein are phosphorylated by a single enzyme species.

Materials and Methods

[γ^{32}P] adenosine triphosphate (sp act >10 Ci/μmol) was obtained from New England Nuclear (Dorval, Que.) and diluted to the required sp. act. with unlabeled crystalline ATP from the Sigma Chemical Co. (St. Louis, Mo.). Casein was obtained from the ICN Pharmaceuticals Inc. (Cleveland, Ohio). The components for polyacrylamide gel electrophoresis were obtained from BioRad Corporation (Mississauga, Ont.), except Amido Black 10B which was purchased from Canlab (Toronto, Ont.).

Red Blood Cell Membrane Preparation

Whole blood from healthy donors was collected from a forearm vein into a plastic syringe containing 0.14 vol acid citrate-dextrose anticoagulant [2]. Erythrocyte ghosts were prepared as described by Fairbanks *et al.* [9] except that 10 mM Tris-HCl buffer, pH 8.0, was used instead of 5 mM phosphate buffer, pH 8.0. Spectrin-depleted vesicles were prepared from fresh ghosts as described by Avruch *et al.* [4] using six strokes of a tight-fitting all-glass homogenizer rather than aspiration through a syringe needle for conversion of the spectrin-depleted membrane to vesicles.

Casein Phosphorylation by Ghosts and Vesicles

Casein (0.4 mg) was phosphorylated at a maximum rate in an incubation mixture containing 20 mM imidazole-HCl buffer, pH 7.4 1 mM $MgCl_2$, 250 mM NaCl, 0.005% saponin (included to maintain ghost permeability, [20]), 100 μM [γ^{32}P] ATP (sp act 20 mCi/mmole) with approximately 75 μg of ghosts or vesicles, in a total volume of 100 μl. After incubation for 15 min at 37 °C, the reaction was terminated by the addition of 1.0 ml of 0.3 N $HClO_4$ containing 5 mM H_3PO_4. 400 μg bovine serum albumin (BSA) were added to samples which did not contain casein to act as a control for nonspecific binding of radioactive material to protein. After cooling on ice for 20 min, the precipitate was collected on glass fiber filters (Whatman 934 AH from O.H. Johns, Toronto, Ont.) and washed five times with 2-ml portions of the $HClO_4$–H_3PO_4 solution and finally with 1 ml of methanol. After drying for 15 min under a heat lamp, the filters were transferred to scintillation vials (Mini-Vial Canatech, Toronto, Ont.), 5 ml of a toluene-based scintillation cocktail containing 5 g PPO and 0.1 g POPOP/liter were added, and the radioactivity was determined using a Beckman liquid scintillation counter. In all cases casein phosphorylation is reported as the difference between the extent of the incorporation of [^{32}P] phosphate in incubations containing casein and those without.

Recovery of Ghosts from Competition Experiment Incubation Mixtures

Since the casein protein overlaps the erythrocyte membrane proteins when fractionated on SDS-PAGE gels (Fig. 1*A* and *B*), it was necessary to recover ghosts, free of casein, after the competition assay incubations. When the wash buffer contained Mg^{2+}, it was found that the casein was not removed from the ghosts (Fig. 1*C*). Washing with 20 mM imidazole-HCl buffer pH 7.4 alone reduced the amount of casein binding, while washing with 20 mM imidazole-HCl buffer 7.4 and 0.3 mM EGTA was most effective (Fig. 1*D*), and after this treatment no casein or [^{32}P] phosphate was detected on gels in the peaks with higher mobility.

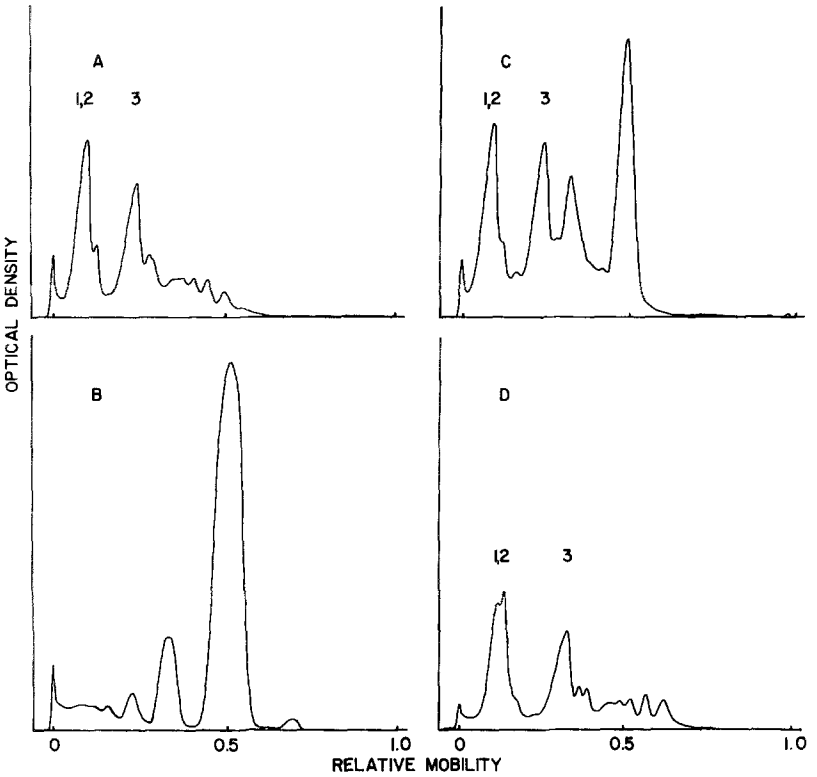

Fig. 1. Densitometer tracings of SDS-PAGE gels upon which were fractionated: (*A*) erythrocyte ghost protein, (*B*) casein, (*C*) erythrocyte ghosts incubated with casein and washed with 20 mM imidazole-HCl buffer, pH 7.4, containing 20 mM $MgCl_2$, and (*D*) erythrocyte ghosts incubated with casein and washed with 20 mM imidazole-HCl buffer, pH 7.4, and 0.3 mM EGTA. Spectrin bands 1 and 2 were resolved on gels but not resolved by densitometer. Numbers in *A*, *C* and *D* indicate the location of band 1 and 2 (spectrin) and band 3

Casein Endogenous Substrate Competition Assay

To examine the competition of casein with the endogenous substrates for phosphorylation, a range of concentrations of casein (0.1 to 5 mg/ml) were included in incubation mixtures consisting of 20 mM imidazole-HCl buffer, pH 7.4, 20 mM $MgCl_2$ 0.005% saponin, 5 μM [γ^{32}P] ATP (sp act 2 Ci/mmol) and approximately 75 μg of ghosts in 100 μl. After incubation for 5 min, the reaction was terminated by the addition of unlabeled ATP to 1 mM (200-fold excess over labeled ATP) and 1 ml of ice-cold 20 mM imidazole-HCl buffer pH 7.4 containing 0.3 mM EGTA and cooling on ice. The membranes were collected by centrifugation at 8,000 rpm for 2.5 min and washed three times by resuspension in the imidazole-EGTA solution and centrifugation. The final ghost pellet was resuspended and the volume adjusted to 100 μl and then mixed with 10 μl 50% sucrose, 10 μl 11% SDS solution containing 5 mM EDTA and 6 mM 2-mercaptoethanol and 2.5 μl of 1% Pyronin Y. The proteins and phospholipids were fractionated on 6% sodium dodecylsul-

phate polyacrylamide gels (SDS-PAGE) and incorporation of [^{32}P] phosphate was determined as described previously [22]. In experiments to examine the effect of casein upon cAMP-dependent phosphorylation the same procedure was used except that cAMP was included at a concentration of 20 μM and [γ^{32}P] ATP (sp act 0.1 Ci/mM) was included at 200 μM. The higher ATP concentration for the assay of the cAMP-dependent protein kinase compared to the cAMP-independent activities was necessary to assure that the phosphorylation of band 4.5 was not affected by changes of ATP concentration. The higher ATP concentration increased the phosphorylation of band 4.5 more than twofold. The necessity for the large increase in ATP concentration may reflect increased hydrolysis of ATP by other enzymes such as the casein kinase (K_m for ATP ~75 μM, *unpublished data*), phospholipid kinase (K_m for ATP 30.8 μM (Fig. 5)) and possibly membranes ATPases.

Protein was determined by the method of Lowry *et al.* [13] using crystalline BSA as the standard protein.

Results

Ionic Conditions for Casein Phosphorylation by Erythrocyte Spectrin-Depleted Vesicles

To compare the cAMP-independent phosphorylation of casein with that of the endogenous substrates, spectrin and band-3 protein, we examined the effect of varying ionic composition of the incubation mixture with casein at 4 mg/ml. Increasing the concentration of $MgCl_2$ from 1 to 20 mM had essentially no effect (10% increase) on casein phosphorylation, a result comparable to band-3 protein phosporylation [8]. Addition of 1 mM $CaCl_2$ to the 1-mM $MgCl_2$ solution reduced casein phosphorylation by 40% in three replicate experiments. This result is similar to band 3-protein phosphorylation, but opposite to spectrin phosphorylation [3]. Addition, however, of increasing amounts of NaCl from 50 to 500 mM while maintaining $MgCl_2$ at 1 mM showed that the optimum NaCl concentration was between 250 and 400 mM (Table 1). Further

Table 1. Effect of Na^+ concentration upon casein phosphorylation by erythrocyte vesicles in the presence of 1 mM $MgCl_2$

Na^+ concentration (mM)	% Activity (compared to 0 NaCl)
50	115
100	138
200	180
250	210
300	226
400	204
500	135

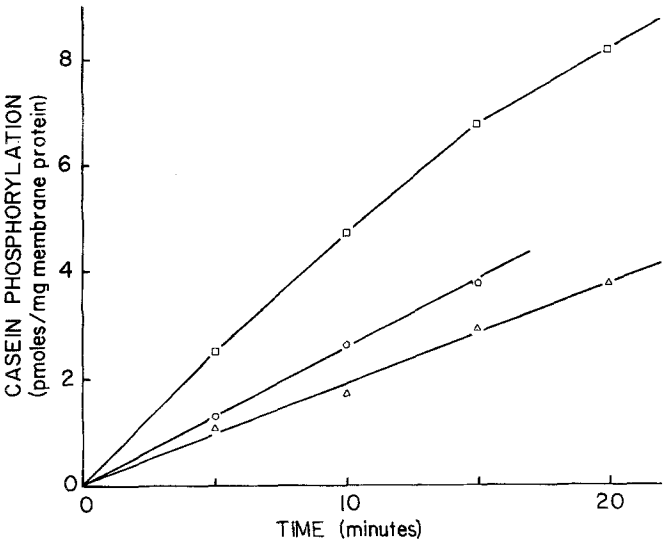

Fig. 2. Time courses of casein phosphorylation by erythrocyte ghosts in the presence of 20 mM $MgCl_2$ (o—o), and erythrocyte vesicles in the presence of 20 mM $MgCl_2$ (△—△) and 1 mM $MgCl_2$ and 250 mM NaCl (□—□). The remainder of the incubation mixture and procedure are described in *Materials and Methods*

experiments in which narrower ranges of NaCl concentrations were studied showed the optimum to be between 250 and 300 mM. This effect of NaCl upon casein phosphorylation is similar to its effect upon spectrin phosphorylation.

The phosphorylation of casein in the presence of 20 mM $MgCl_2$ or 1 mM $MgCl_2$ and 250 mM NaCl was found to be close to linear for at least 15 min, and the activity with the latter conditions was approximately twice that of the former (Fig. 2).

Phosphorylation of Casein by Erythrocyte Ghosts

We have found that the rate of phosphorylation of casein by erythrocyte ghosts is only 40% higher than that with vesicles under the same incubation conditions and the extent is close to linear with respect to time (Fig. 2). Thus, while the rate of phosphorylation of casein by vesicles was 196 ± 2.2 pmol/min/mg, the rate of phosphorylation by the ghosts from which the vesicles were prepared was 272 ± 5.0 pmol/min/mg. Since the membrane undergoes considerable disruption and some loss of organ-

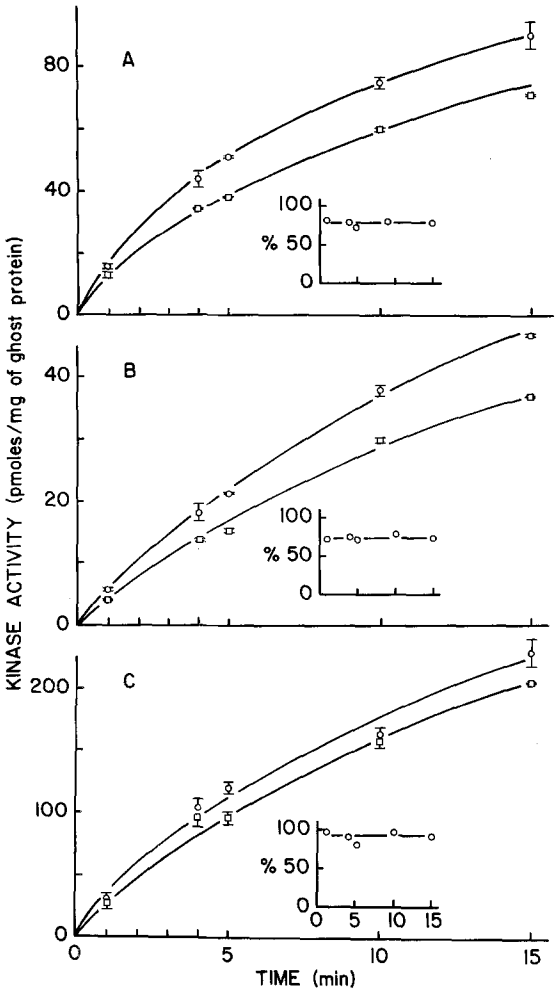

Fig. 3. Time courses of the phosphorylation of spectrin (*A*), band-3 protein (*B*), and phospholipid (*C*) in the absence (o—o) and presence (□—□) of casein (1 mg/ml). Competition assays were performed as described in *Methods* and incorporation of [^{32}P] phosphate determined on SDS-PAGE gels. Insets show the activity remaining in samples incubated with casein expressed as a percentage of corresponding samples incubated without casein at each time

ization during preparation of vesicles, the loss in activity does not seem excessive. Alternatively, it is possible that some of the casein kinase is lost in the preparation of vesicles resulting in lower activity although the low ionic strength of the solution used would not be expected to elute casein kinase.

Competition of Casein with the Endogenous Substrates for Phosphorylation cAMP-Independent Phosphorylation

The time course of phosphorylation of spectrin, band-3 protein and phospholipid was examined to determine whether the competition of casein with the endogenous substrates varied with time and whether the rate of phosphorylation decreased at longer time periods, as a result of ATP consumption. Figure 3 shows that the rates of phosphorylation of spectrin and band-3 protein in the presence of casein at a concentration of 1 mg/ml were not greatly reduced at 15 min and the extent of competition by casein was uniform up to 15 min. A 5-min incubation period was deemed appropriate on the basis of these data.

Table 2 shows the rates of phosphorylation of spectrin, band-3 protein and phospholipids in the presence of a range of concentrations of casein. With the exception of the sample in which spectrin phosphorylation was assayed in the presence of 0.1 mg/ml casein, the phosphorylation of the three substrates is significantly lower in the presence of casein. When the phosphorylation of spectrin and band-3 protein in the presence of the various casein concentrations was compared, it was further found that these two reductions were highly correlated (correlation coefficient $(r) = 0.92$, significant at $p < 0.001$). In contrast, the reductions of spectrin and band-3 phosphorylation were not correlated with the reduction of

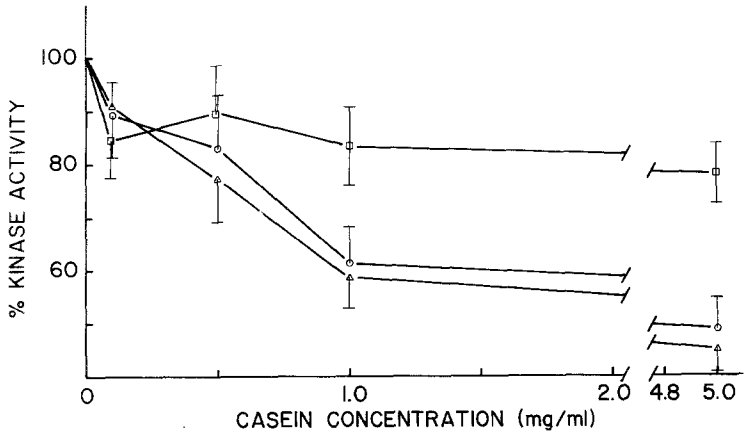

Fig. 4. Plots of percentage kinase activities remaining in the presence of increasing concentrations of casein compared to the activity in the absence of casein. Spectrin phosphorylation (○—○), band-3 protein phosphorylation (△—△), and phospholipid phosphorylation (□—□)

Table 2. Effect of casein upon the cAMP-independent phosphorylation of spectrin, band-3 protein, and phospholipid in the presence of 5 μM ATP

Casein concentration (mg/ml)	Kinase activity[a] (pmole/min/mg ghost protein)					
	Spectrin	P^b	Band 3	P^b	Phospho-lipid[d]	P^b
0 (36)[c]	21.2±1.21		5.50±0.27		8.36±0.43	
0.1 (20)	19.0±1.73	NS	4.99±0.41	<0.05	7.08±0.57	<0.001
0.5 (14)	17.6±2.09	<0.005	4.24±0.44	<0.001	7.48±0.74	<0.05
1.0 (18)	13.1±1.43	≪0.001	3.22±0.31	≪0.001	6.96±0.61	<0.005
5.0 (20)	10.4±1.26	≪0.001	2.48±0.23	≪0.001	6.55±0.48	<0.001

[a] Activities are presented as the mean ± the SEM.

[b] Based upon a comparison of the activity at each concentration with the activity in the absence of casein using a paired *t* test.

[c] Numbers in brackets are the number of independent experimental samples upon which the mean was based. In most experiments controls were done in quadruplicate and experimental samples in duplicate.

[d] Label present in the 2-cm portion, sample of SDS-PAGE gels immediately behind the tracking dye. Destaining of gels was sufficient to remove any unreacted [γ^{32}P] ATP of [^{32}P] phosphate which would also be present in this portion of the gels.

phospholipid phosphorylation ($r=0.20$, and $r=0.27$, respectively, not significant).

Figure 4 illustrates the correlation between the reduction of phosphorylation of spectrin and band-3 protein and the absence of such correlation with the reduced phospholipid phosphorylation. It also shows that, although the reduction of actual activities is different (Table 2) (due to the fact that spectrin protein kinase activity is considerably higher than band-3 protein kinase activity), the reductions calculated as percentages are almost identical.

cAMP-Dependent Phosphorylation

To examine the specificity of the competition of casein with endogenous substrates, the effect of increasing amounts of casein upon the cAMP-dependent phosphorylation of band 4.5 was examined. This substrate has an advantage over other cAMP-dependent protein kinase substrates because it is not closely associated in gels with cAMP-independent protein kinase substrates (e.g., band 2.1 is very close to band 2).

As shown in Table 3, at higher concentrations of ATP the extent of spectrin phosphorylation, and the response to casein is comparable

Table 3. Effect of casein upon the cAMP-independent phosphorylation of spectrin and cAMP-dependent phosphorylation of band-4.5 protein and phospholipid in the presence of 200 μM ATP

Casein concentration (mg/ml)	Kinase activity[a] (pmole/min/mg ghost protein)			
	Spectrin −cAMP	Band 4.5		Phospholipid +cAMP[b]
		−cAMP	+cAMP[b]	
0	15.0 ±0.28	6.15±0.03	8.39±0.11	73.5±3.1
0.1	14.9 ±0.09	6.22±0.13	8.12±0.13	73.5±2.9
0.5	12.4 ±0.20	5.70±0.01	8.38±0.02	66.0±1.3
1.0	11.4 ±0.12	5.48±0.0	9.64±0.04	63.7±3.3
5.0	6.66±0.04	4.10±0.16	2.82±0.17	46.1±4.1

[a] Data are means of two independent samples and are reported as the mean ± the SD of the mean; which in this case is half the difference between the two sample values.

[b] cAMP activity is the difference between the activity in the presence of cAMP and in the absence of cAMP.

to that at lower ATP concentrations (Table 2). In contrast, the cAMP-dependent phosphorylation of band 4.5 is not reduced in the presence of 0.1, 0.5 and 1.0 mg/ml casein (Table 3). The cAMP-dependent phosphorylation of band 4.5, however, is reduced in the presence of 5 mg/ml casein.

The phosphorylation of phospholipids was also reduced by increasing concentrations of casein, although the reduction in the presence of casein concentrations up to 1.0 mg/ml was only 13%, whereas the reduction by 5 mg/ml casein was 37%.

The possibility that casein caused elution of the spectrin and band-3 protein kinase and thus reduction of activity was examined by pre-incubating erythrocyte ghosts with concentrations of casein from 0 to 5 mg/ml in the normal incubation mixture from which ATP was omitted. The casein and ghosts were recovered by centrifugation and separately incubated with [γ^{32}P] ATP. There was no increase in phosphorylation of casein in the supernate of incubation mixtures that contained casein. Assay of the washed ghosts also indicated that there was no loss of kinase activity in ghosts pre-incubated with casein; this would have been expected if casein had caused elution of kinase.

Casein Phosphorylation in Competition Experiments

To determine whether the progressive reduction of phosphorylation of the endogenous substrates in response to the increasing amounts of

Table 4. Phosphorylation of casein recovered from the supernate of the competition assay

Casein concentration (mg/ml)	[^{32}P] Phosphate incorporation into casein[a]	
	Rate (pmol/min/mg ghost protein)	Total (pmol/100 µl incubation mixture in 5 min incubations)
0.1	27.1 ± 4.0[b]	10.8 ± 1.60
0.5	57.0 ± 5.7	22.8 ± 2.3
1.0	77.2 ± 6.3	30.9 ± 2.5
5.0	99.1 ± 10.3	39.6 ± 4.1

[a] These data are the means of four separate experiments in which phosphorylation in the absence and presence of casein was determined and the difference reported as casein phosphorylation.

[b] Mean ± SEM.

casein was due to the increasing consumption of ATP by the phosphorylation of casein, we determined the extent of phosphorylation of casein, recovered from competition experiment supernates. Casein in 1 ml of the supernate was precipitated by addition of concentrated $HClO_4$ and concentrated H_3PO_4 to give a final concentration of 0.3 N $HClO_4$ and 5 mM H_3PO_4, and was collected and washed on filters as described for the routine casein phosphorylation assay.

There was only a fourfold increase in the rate or total amount of [^{32}P] phosphate labeling of casein in response to 50-fold increase of the amount of casein present (Table 4). Furthermore, when the total amount of phosphorylation of casein in a 100 µl incubation mixture containing the maximum amount of casein (5 mg/ml) was compared with the amount of available ATP (500 pmol), the result indicated that a maximum of only approximately 8% of the ATP was used up by casein phosphorylation.

Discussion

These data support the suggestion that spectrin and band-3 protein are phosphorylated by the same protein kinase which also phosphorylates casein. It is, however, possible that casein causes the reduction of phosphorylation of the two protein substrates by several other mechanisms: competition for ATP, physically preventing access of the endogenous substrates to the enzymes, or by perturbing the membrane organization and indirectly affecting the protein kinase and/or substrates.

Phosphorylation of casein by erythrocyte ghosts required less than 8% of the available ATP. This small decrease in the available ATP should not markedly influence the extent of phosphorylation of the endogenous proteins. This was confirmed by the continued phosphorylation of the endogenous substrates beyond 5 min (Fig. 3) and the similarity of the results obtained in the presence of 5 and 200 μM ATP.

If casein were sterically hindering the phosphorylation of the endogenous substrates, either by remaining associated with the enzyme or near the enzyme, phosphorylation of other casein molecules would also be reduced. However, casein is phosphorylated by the protein kinase(s) at a higher rate than the endogenous proteins (Table 4 compared to 2). Therefore, this alternative seems improbable.

It is unlikely that casein could be phosphorylated by the phospholipid[1] kinase due to the difference in chemical nature of the two molecules. Under the ionic conditions of the assay, the K_m for ATP of phospholipid kinase is 30.8 μM (Fig. 5*C*). Thus, the ATP concentration in the competition assays (5 μM) would be rate-limiting for phospholipid phosphorylation, and reductions in the ATP concentration would be expected to affect the extent of phospholipid phosphorylation. The observation that the reduction of phospholipid phosphorylation parallels the extent of casein phosphorylation also indicates it is likely due to competition for ATP. Since the reduction of phospholipid phosphorylation is not correlated with that of spectrin and band 3-protein, the results support the suggestion that the reduction of protein kinase activity is due to direct competition of the casein with the endogenous substrates for phosphoylation.

The results of casein competition with the cAMP-dependent phosphorylation of band 4.5 confirm the specificity of casein phosphorylation by cAMP-independent protein kinase(s). Thus, when spectrin phosphorylation is clearly reduced, (0.5 and 1.0 mg/ml casein) no decrease is seen in band-4.5 cAMP-dependent phosphorylation. The reduction of band-4.5 cAMP-dependent phosphorylation by casein at 5 mg/ml may be due to either reduction of available ATP to levels which are limiting for the kinase which phosphorylates band 4.5 or competition with the endogenous substrate by casein. With the high ATP concentrations a slight stimulation of casein phosphorylation (10%) was noted with cAMP (data

[1] Under the conditions of these assays we found only two phospholipids, di- and tri-phosphoinositide labeled with [^{32}P] phosphate (*unpublished*) which is consistent with the results of Buckley [6] and Garret and Redman [10]. Since we did not isolate these compounds in these experiments, we will refer to their labeling as that of phospholipids.

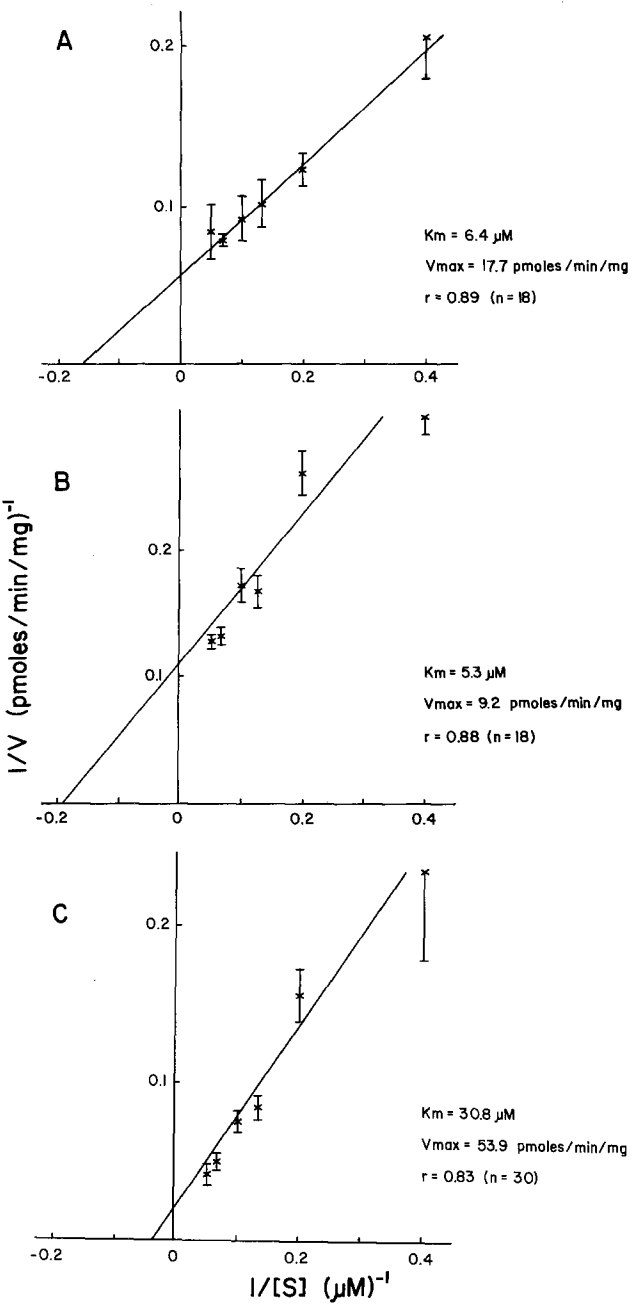

Fig. 5. Linewaver-Burk plots of the rate of phosphorylation of spectrin (*A*), band-3 protein (*B*), and phospholipid (*C*) in response to ATP concentrations ([*S*]) from 2.5 to 20 μM. Incubation was at 37 °C for 5 min in a mixture identical to the casein competition assay mixture except casein was omitted. Data points are the means ± SEM of three experiments in *A* and *B* and four experiments in *C*. The *n* value is the total number of independent samples in the group of experiments. The number of samples at one concentration of ATP varied from 2 (in one case) to 6 (normally 4). The K_m and V_{max} values were determined by linear regression analysis upon the individual values not the means, although the latter gave an almost identical result

not presented). Thus, casein may serve as a poor (low affinity) substrate for the cAMP-dependent protein kinase when present at high concentrations in the presence of high concentrations of ATP.

The observation that spectrin and band-3 protein phosphorylation are reduced identically strongly supports the suggestion made by Avruch *et al.* [4] that both are phosphorylated by the same enzyme. If two separate enzymes were present, the existence of a relationship between the affinities and accessibility of both enzymes for casein, such that spectrin and band-3 protein phosphorylation would be equally reduced, would seem unlikely, although our data do not eliminate such a possibility.

The investigation of conditions to minimize casein binding to ghosts in the competition assay provides evidence of the similarity of the interaction of casein with the ghost membrane to that of spectrin. Thus, the conditions which minimize casein binding to ghosts (low ionic strength and presence of EGTA) are also those known to promote spectrin release (low ionic strength and absence of divalent cations). In the competition assay washes, spectrin was not lost, presumably due to the short period of exposure to the ionic conditions and to the use of 4 °C rather than 37 °C, which is used for spectrin elution.

Changes in the interactions of protein kinase(s) with the endogenous substrates would likely be reflected in changes in the ability of casein to compete for phosphorylation. Thus, this system may also provide a means to study both the interactions of membrane-bound protein kinase(s) with spectrin, and band 3 and the effects of the membrane environment upon them *in situ*.

This research was supported by a Grant-in-Aid from the Medical Research Council of Canada (The MRC Group in Developmental Neurobiology) to MPR.

The authors wish to thank Miss C. Habinski for typing the manuscript and Drs. Brown, Carlone, and Werstiuk for their comments and criticisms of the manuscript.

JDV is a Post-doctoral Fellow of the Muscular Dystrophy Association of Canada. JB is a recipient of a Muscular Dystrophy Association of Canada Summer Studentship.

References

1. Appel, S.H., Roses, A.D. 1976. Membrane biochemical studies in myotonic muscular dystrophy. *In:* Membranes and Disease L. Bolis, J.F. Hoffman and A. Leaf, editors. pp. 183–195. Raven Press, New York
2. Aster. R.H., Jandl, J.H. 1964. Platelet sequestration in man. I. Methods. *J. Clin. Invest.* **43**:843

3. Avruch, J., Fairbanks, G. 1974. Phosphorylation of endogenous substrates by erythrocyte membrane protein kinases. I. A monovalent cation-stimulated reaction. *Biochemistry* **13**:5507
4. Avruch, J., Fairbanks, G., Crapo, L.W. 1976. Regulation of plasma membrane protein phosphorylation in two mammalian cell types. *J. Cell Physiol.* **89**:815
5. Bruni, A., Van Dijck, P.W.M., DeGier, J. 1975. The role of phospholipid acyl chains in the activation of mitochondrial ATPase complex. *Biochim. Biophys. Acta* **406**:315
6. Buckley, T.B. 1977. Properties of human erythrocyte phosphatidylinositol kinase and inihibition by adenosine, ADP and related compounds. *Biochim. Biophys. Acta* **498**:1
7 Coleman, R. 1973. Membrane-bound enzymes and membrane ultrastructure. *Biochim. Biophys. Acta* **300**:1
8. Fairbanks, G., Avruch, J. 1974. Phosphorylation of endogenous substrates by erythrocyte membrane protein kinases. II. Cyclic adenosine monophosphate-stimulated reactions. *Biochemistry* **13**:5514
9. Fairbanks, G., Steck, T.L., Wallach, D.F.H. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane *Biochemistry* **10**:2606
10. Garrett, R.J.B., Redman, C.M. 1975. Localization of enzymes involved in polyphosphoinositide metabolism on the cytoplasmic surface of the human erythrocyte membrane. *Biochim. Biophys. Acta* **382**:58
11. Hosey, M.M., Tao, M. 1977. Selective phosphorylation of erythrocyte membrane proteins by the solubilized membrane protein kinases. *Biochemistry* **16**:4578
12. Hosey, M.M., Tao, M. 1977. Protein kinases of rabbit and human erythrocyte membranes. Solubilization and characterization. *Biochim. Biophys. Acta* **482**:348
13. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**:265
14. Missirlis, Y.F., Kohn, I., Vickers, J.D., Rathbone, M.P., Chui, D.H., McComas, A.J., Brain, M.C. 1977. *In:* Erythrocyte Membranes: Recent Clinical and Experimental Advances. W.C. Kruckeberg, J.W. Eaton, and G.J. Brewer, editors. pp. 189–200. Alan R. Liss, New York
15. Roelofsen, B., Schatzmann, H.J. 1977. The lipid requirement of the $(Ca^{2+}+Mg^{2+})$-ATPase in the human erythrocyte membrane, as studied by various highly purified phospholipases. *Biochim. Biophys. Acta* **464**:17
16. Roses, A.D., Appel, S.H. 1973. Protein kinase activity in erythrocyte ghosts of patients with myotonic muscular dystrophy. *Proc. Nat. Acad. Sci. USA* **70**:1855
17. Roses, A.D., Herbstreith, M.H., Appel, S.H. 1975. Membrane protein kinase alteration in Duchenne muscular dystrophy. *Nature (London)* **254**:350
18. Semenuk, M., Vickers, J., Rathbone, M.P., Brain, M.C. 1977. The effect of cholesterol-loading of red blood cells (RBCs) on membrane-bound protein kinase (PKase). *Clin. Res.* **25**:695A
19. Sha'afi, R.I., Rodan, S.B., Hintz, R.L., Fernandez, S.M., Rodan, G.A. 1975. Abnormalities in membrane microviscosity and ion transport in genetic muscular dystrophy. *Nature (London)* **254**:525
20. Steck, T.L. 1974. Preparation of impermeable inside-out and right-side-out vesicles from erythrocyte membranes. *In:* Methods in Membrane Biology. E. Korn, editor. Vol. 2, pp. 245–281. Plenum, New York
21. Vickers, J.D., McComas, A.J., Rathbone, M.P. 1977. Altered activity and temperature response of membrane-bound protein kinases in erythrocytes of patients with myotonic muscular dystrophy. *Neurosci. Abstr.* **3**:224
22. Vickers, J.D., McComas, A.J., Rathbone, M.P. 1978. Alterations of membrane phosphorylation in erythrocyte membranes from patients with Duchenne muscular dystrophy. *Can. J. Neurol. Sci.* **5**:437

23. Vickers, J.D., McComas, A.J., Rathbone, M.P. 1979. Myotonic muscular dystrophy: Abnormal temperature response of membrane phosphorylation in erythrocyte membranes. *Neurology* **29**:791
24. Vickers, J.D., Rathbone, M.P. 1977. Effect of erythrocyte membrane cholesterol on a membrane protein kinase activity. *Proc. Can. Fed. Biol. Soc.* **20**:65
25. Warren, G.B., Housley, M.D., Metcalf, J.C., Birdsall, N.J.M. 1975. Cholesterol is excluded from the phospholipid annulus surrounding an active calcium transport protein. *Nature (London)* **255**:684