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Phosphorylation of ventricular sarcolemmal membranes does not alter binding properties of nitrendipine

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Isoproterenol increased contractility in isolated cat papillary muscles 2-fold with an EC_{50} of $6.3 \cdot 10^{-8}$ M. Nifedipine ($3 \cdot 10^{-7}$ M) reduced contractility in control muscles by 43%; however, inotropic state was restored by isoproterenol with a comparable EC_{50} of $5 \cdot 10^{-8}$ M. To test the hypothesis that this effect might result from cAMP-dependent phosphorylation of a Ca^{2+} channel-associated protein, [^{3}H]nitrendipine binding was used to probe the high-affinity 1,4-dihydropyridine site in either phosphorylated or dephosphorylated sarcolemmal vesicles. K_d and B_{max} values for binding to phosphorylated sarcolemmal vesicles (0.14 ± 0.027 nM and 479 ± 62 fmol/mg protein, respectively) were not significantly different from control values ($P > 0.4$). Similarly, dephosphorylation of sarcolemmal vesicles did not alter binding parameters. These data demonstrate that phosphorylation of sarcolemmal vesicles neither alters the binding affinity for [^{3}H]nitrendipine nor promotes an interconversion of dihydropyridine-binding sites from high to low affinity or vice versa. While phosphorylation may regulate the slow Ca^{2+} channel, this is not reflected as changes in [^{3}H]nitrendipine-binding parameters determined in vitro. Furthermore, the cyclic AMP-dependent phosphorylation state of sarcolemmal proteins does not appear to account for wide variations (more than 100-fold) between K_d values from binding studies and IC_{50} values determined in pharmacological investigations.

Introduction

Calcium antagonists were originally postulated by Fleckenstein [1] to be a class of drugs that produce relatively specific inhibition of the slow Ca^{2+} channel in plasma membranes. Direct experimental evidence in support of this hypothesis has recently been reported [2]. In the myocardium, the functional consequence of such inhibition is a pronounced negative inotropic effect. Interestingly, this effect can be completely reversed by activation of β-adrenoceptors [3].

The mechanism whereby β-agonists reverse the actions of calcium antagonists is unknown; however, one of the most viable hypotheses includes a protein phosphorylation-mediated reaction which results in an increase in Ca^{2+} uptake via the slow channel and thus a stimulation of contractility [4–7]. It is known that adrenergic and cholinergic agonists have profound effects on the slow calcium current of cardiac muscle. Catecholamines and cAMP enhance the slow current [8,9] while it is attenuated by muscarinic stimulation [10]. These data are significant in that muscarinic agonists appear to specifically antagonize cAMP-mediated events without altering Ca^{2+} or α_1-receptor responses [11–13]. In addition, microinjection of purified catalytic subunit of cAMP-dependent protein kinase enhances the slow inward current, and this is associated with an increase in the amplitude of contraction and the rate of relaxation [6,14] effects which are not further stimulated by

epinephrine. By contrast, microinjection of the regulatory subunit of protein kinase attenuates the slow inward current and this effect is reversed by epinephrine [6]. Collectively, these data support the hypothesis that the slow inward calcium current in the heart is regulated by phosphorylation of membrane proteins. Indeed, cardiac sarcolemmal vesicles contain both cAMP-dependent protein kinase and protein kinase substrates [15,16] and selected sarcolemmal vesicle proteins are phosphorylated in response to catecholamines [17]. Therefore, it is reasonable to suppose that phosphorylation of either a slow Ca^{2+} channel protein or a regulatory protein juxtaposed to the Ca^{2+} channel could activate a silent channel or potentiate Ca^{2+} movement through a functional pore.

To determine if isoproterenol's reversal of the negative inotropic effects of nifedipine could be explained by the cAMP-dependent phosphorylation of a calcium channel-associated protein, [^{3}H]nitrendipine binding was used to probe the high-affinity 1,4-dihydropyridine-binding site in either phosphorylated or dephosphorylated ventricular sarcolemmal vesicles. Also, since the EC_{50} values for pharmacological effects in the heart are as much as 100-fold the dissociation constants obtained from binding studies [18], it seemed possible that this discrepancy could be explained by in vitro (binding studies) and in vivo (pharmacology) differences in phosphorylation state of sarcolemmal vesicle proteins.

Methods and Materials

Papillary muscle studies. Cats of either sex were anesthetized with methoxyflurane, their hearts immediately removed, and the papillary muscles dissected and suspended in individual muscle-baths. A 27-gauge hook secured the muscle to an electrode mounted in the bottom of the bath and a silk thread attached the tendon to a Statham isometric transducer. Baths contained Krebs-Henseleit solution (37.5°C, bubbled with 95% O_2/5% CO_2) of the following millimolar composition: NaCl, 118; KCl, 4.5; $CaCl_2$, 2.5; KH_2PO_4, 1.1; $MgSO_4$, 1.2; $NaHCO_3$, 25 and glucose, 11. A baseline tension of 1.0 g was applied to each tissue. Muscles were stimulated to contract by administering square-wave pulses of constant voltage (2.0 ms in duration, 12 per min, 20% above threshold voltage) delivered through the hook electrode and a second electrode positioned near the top of the muscle; contractions were recorded on a Grass polygraph. Muscles were equilibrated for 60 min prior to drug treatment. To establish cumulative dose-response relationships, drugs were added to baths and allowed to produce maximal responses before the addition of the next higher concentration. Basal contractility was not altered by $5 \cdot 10^{-7}$ M propranolol – a concentration of antagonist that caused a 550-fold rightward shift in the EC_{50} for isoproterenol.

Binding studies Cardiac sarcolemmal vesicles were prepared from ventricles of pentobarbital-anesthetized mongrel dogs according to the methods of Jones et al. [19]. The final pellet was resuspended in 0.25 M sucrose/10 mM histidine (pH 7.5) to a protein concentration of 2–4 mg/ml and stored frozen at −80°C. Protein concentration was determined by the method of Lowry et al. [20] using bovine serum albumin (fraction V) as the standard.

On the day of a binding experiment, vesicles were thawed, diluted with 3 vol. deionized water and centrifuged for 15 min in a Beckman airfuge. The pellet was resuspended in the appropriate buffer for phosphorylation or dephosphorylation (see below) and treated with alamethicin (1.0 mg/mg sarcolemmal vesicle protein) for 20 min in order to render the vesicles permeable [19].

For binding experiments, 10-μl aliquots of either phosphorylated or dephosphorylated cardiac sarcolemmal membranes or their appropriate controls (approx. 20 μg vesicle protein) were incubated with varying concentrations of [^{3}H]-nitrendipine in 2 ml 50 mM Tris-HCl (pH 7.4). Incubations were carried out in the dark for 60 min at 30°C. Control experiments demonstrated that binding equilibrium was attained under these conditions (data not shown). Bound radioligand was separated from free by rapid vacuum filtration using Whatman GF/B filters. Filters were washed three times with 5-ml aliquots of sodium phosphate (pH 7.4) at 3–5°C, and radioactivity retained by the filter was determined in 10 ml PCS (Amersham) at a counting efficiency of 30–40%. Specific binding was defined as bound radioactivity that was displaceable by 1.0 μM nifedipine.

Phosphorylation / dephosphorylation of purified sarcolemmal vesicles. To phosphorylate membrane proteins, vesicles (2.0 mg/ml) were incubated for 20 min at 23°C in buffer containing 1.0 mg alamethicin/mg protein, 20 mM Tris-malate (pH 7.0), 15 mM potassium phosphate, 100 mM KCl, 0.1 mM isobutylmethylxanthine and 5 mM $MgCl_2$. The reaction was started by adding 25 μM [γ-^{32}P]ATP, 2.5 μCi/nmol, and 10 μM cAMP. At indicated times, aliquots of sarcolemmal vesicles were mixed 2 : 1 with 0.1 M Tris-HCl (pH 8.6), 2% sodium dodecyl sulfate (SDS), 0.2% Bromophenyl blue and 10% sucrose. Samples were heated at 95°C for 10 min, cooled to 23°C, and 2-mercaptoethanol added to a final concentration of 0.75 M. Proteins were resolved by SDS-polyacrylamide gel electrophoresis based on a published procedure [21]; gels were stained, dried and subjected to autoradiography [22]. Incorporation of ^{32}P into proteins was determined either by scanning autoradiographs or liquid-scintillation spectrometry of cut-out bands. Phosphorylation was blocked in a concentration-dependent manner by 15–4500 μg/ml of the heat-stable inhibitor of protein kinase [23] (data not shown).

Although it is unlikely that purified sarcolemmal vesicles are significantly phosphorylated, it was of interest to determine if endogenous phosphate could be removed. To study dephosphorylation, vesicles were maximally phosphorylated following a 10-min reaction in buffer containing 1.0 mg alamethicin/mg protein, 20 mM Tris-malate (pH 7.0), 15 mM potassium phosphate, 0.1 mM isobutylmethylxanthine, 100 mM KCl, 10 μM cAMP and 250 μM [γ-^{32}P]ATP (1.0 μCi/nmol). After washing two times to remove ATP, crude rabbit skeletal muscle phosphatase [24] was added and the reaction was allowed to proceed at 30°C; aliquots were removed between 0 and 2 h to quantify ^{32}P content of proteins.

To study [^{3}H]nitrendipine binding in phosphorylated sarcolemmal vesicles, vesicles were phosphorylated as previously described; control sarcolemmal vesicle incubations contained no cAMP or ATP. Following a 10-min incubation at 30°C, vesicles were pelleted in a Beckman airfuge (15 min centrifugation) and resuspended to a final concentration of 2.0 mg/ml in 50 mM Tris-HCl (pH 7.4). Control experiments demonstrated that these conditions resulted in a maximal membrane phosphorylation that was maintained throughout the binding experiments. Dephosphorylated sarcolemmal vesicles for binding studies were obtained by incubating vesicles with crude rabbit skeletal muscle phosphatase for 2 h at 30°C while control vesicles contained no phosphatase. Sarcolemmal vesicles were collected by airfuge centrifugation and prepared as described above for binding studies. Experiments with vesicles that had been maximally phosphorylated [25] demonstrated that treatment with phosphatase removed 98% of the ^{32}P from the 55 and 12 kDa bands and 58% from the 15 kDa band.

Analysis of data. Control and experimental [^{3}H]nitrendipine binding curves (phosphorylated or dephosphorylated sarcolemmal vesicles) were generated on the same day so that 'paired' statistical analysis could be performed. Each isotherm consisted of binding determinations (performed in triplicate) at nine concentrations of [^{3}H]nitrendipine ranging from 0.003 to 1.0 nM. K_d and B_{max} values were obtained by nonlinear least-squares analysis using the LIGAND program [26]. Differences between experimental and control values for either K_d or B_{max} were analyzed for statistical significance by utilizing an F-test to compare goodness of fit as described in Ref. 26. Binding studies (control vs. experimental) were performed a minimum of two times; results from a typical experiment along with the corresponding statistical analysis are presented in the text. Student's t-test was employed for statistical analysis of data other than binding parameters. P values of less than 0.05 were accepted as statistically significant.

Materials. Radioisotopes including [^{3}H]-nitrendipine (70–90 Ci/mmol) were purchased from New England Nuclear and the hydrochloride salts of DL-isoproterenol and DL-propranolol were obtained from Sigma. Nifedipine was kindly supplied by Miles Laboratories, New Haven, CN.

Results

Nifedipine-isoproterenol interactions and cardiac contractility

Isoproterenol caused the well-known and anticipated increase in the inotropic state of cat papillary muscles with an EC_{50} value of $6.3 \cdot 10^{-8}$

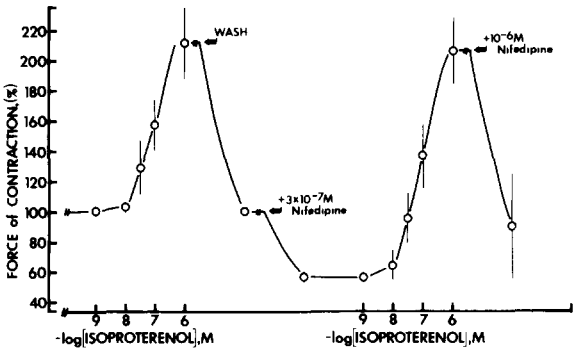

Fig 1 Concentration-dependent effects of nifedipine and isoproterenol on cardiac contractility Experiments were carried out in isolated cat papillary muscles Values represent the mean ± S E of 5–7 experiments

M (Fig. 1). This response occurred in a concentration-dependent manner and was attenuated by propranolol (data not shown). Effects of nifedipine ($3 \cdot 10^{-7}$ M) on basal and isoproterenol-stimulated contractility are shown in Fig. 1. Although nifedipine reduced contractility by 43%, isoproterenol reversed this effect with an EC_{50} value of $5 \cdot 10^{-8}$ M. That is, the percent increase in contractility in response to each concentration of isoproterenol was comparable in either the presence or absence of nifedipine. When maximally stimulated muscles were exposed to $1 \cdot 10^{-6}$ M nifedipine, contractility returned to control values. Addition of $1 \cdot 10^{-6}$ M nifedipine to control muscles resulted in a loss of contraction within 1 min (data not shown).

Phosphorylation / dephosphorylation of sarcolemmal vesicles, stability of ^{32}P-labeled phosphoproteins

Cyclic AMP-dependent phosphorylation of sarcolemmal vesicles by endogenous protein kinases occurred in a time-dependent manner and was maximal between 1.5 and 3.0 min (Fig. 2A). Incorporation of ^{32}P into bands with molecular weights of 15 000 and 12 000 was evident following resolution by SDS-polyacrylamide gel electrophoresis (a 24 kDa protein is phosphorylated and converted to a 12 kDa species by heating samples prior to resolution [22,25]). A 55 kDa band was also apparent with longer film exposure.

To determine if proteins remained phosphorylated during binding studies, vesicles were phosphorylated for 10 min in the presence of cAMP + alamethicin, washed, resuspended and incubated under conditions used to quantify binding. Aliquots were removed between 0 and 120 min, heated (95°C, 5 min), and proteins were resolved by SDS-polyacrylamide gel electrophoresis. The autoradiograph in Fig. 2B clearly demonstrates that ^{32}P incorporation is constant during the 60-min binding studies.

During most purification processes *, phosphoproteins become dephosphorylated – probably as a result of removing ATP and the persistance of phosphatase activity. Nevertheless, to study [^{3}H]nitrendipine binding under conditions representing the maximally dephosphorylated state, it was of interest to demonstrate that sites retaining phosphate following sarcolemmal vesicle purification could be dephosphorylated prior to binding studies. Maximally phosphorylated sarcolemmal vesicles (+cAMP, ATP, $MgCl_2$, isobutylmethylxanthine, alamethicin [25,27]) were incubated with a 1 : 5 (v/v) extract of crude rabbit skeletal muscle phosphatase **. As shown in Fig. 2C, dephosphorylation of the 55 and 12 kDa proteins occurred in a time-dependent manner and was nearly complete after 2 h, while the 15 kDa protein retained 42% of its initial ^{32}P. The rate of dephosphorylation was also dependent on the concentration of phosphatase present (data not shown). These experiments demonstrate that if any phosphate were retained after membrane isolation, it would in all likelihood be significantly removed by this procedure. Vesicles prepared to study nitrendipine binding under dephosphorylated conditions were not incubated under phosphorylating conditions but rather were treated only with phosphatase.

Because approx. 80% of the vesicles are right-side-out and tightly sealed (unpublished data), added ATP and cAMP do not readily have access to phosphorylation sites located on the inside of sarcolemmal vesicles. In order to phosphorylate all sites potentially associated with the slow calcium channel, sarcolemmal vesicles were pretreated with

* Phosphatase was purified up to, and including the ammonium sulfate precipitation step

** A 1 . 10 dilution of this preparation removed 80% of the phosphate from 2.0 mg/ml phosphorylase *a* in 10 min

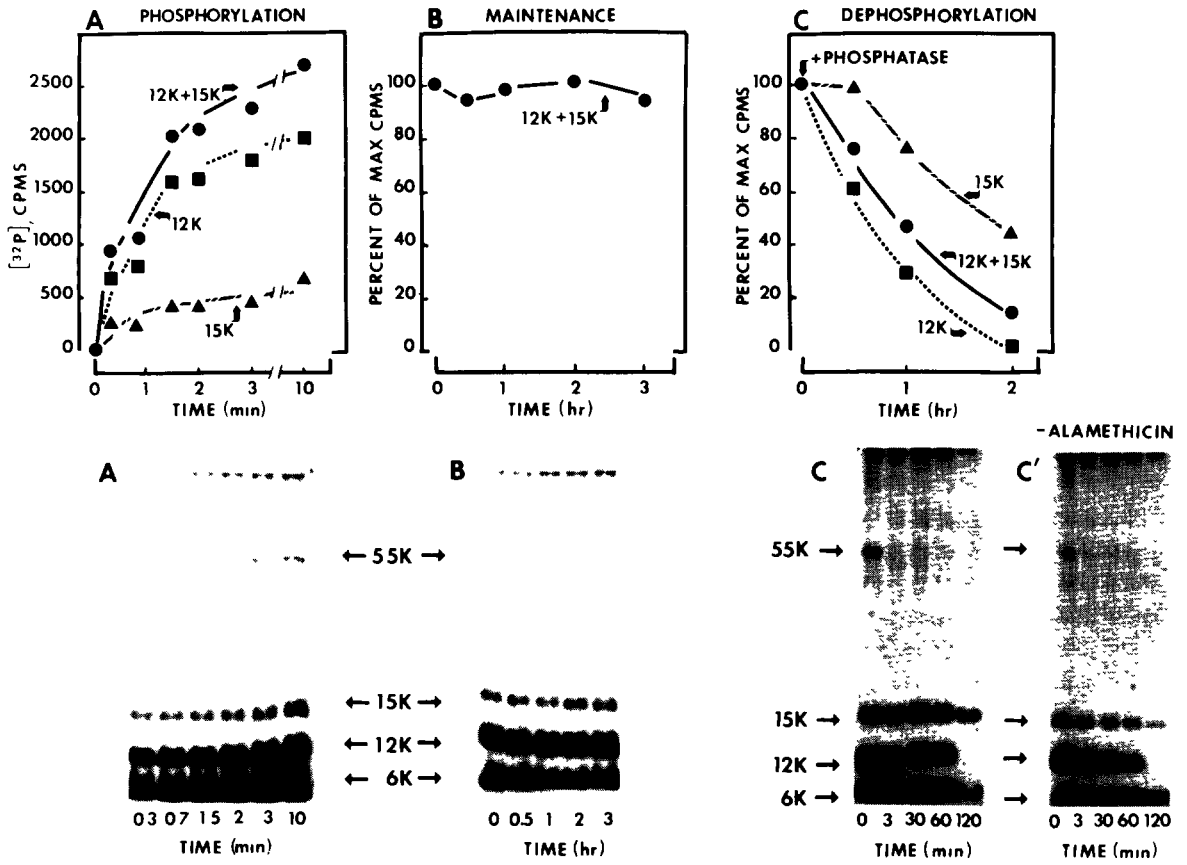

Fig. 2. Phosphorylation of cardiac sarcolemmal vesicles. Panel A: Purified sarcolemmal vesicles were preincubated with alamethicin (1.0 mg/mg protein) and phosphorylated following addition of cAMP and [^{32}P]ATP At 10 min, sarcolemmal vesicles were washed by centrifugation and resuspended in either the presence (panel C) or absence (panel B) of phosphoprotein phosphatase. Dephosphorylation of sarcolemmal vesicles phosphorylated in the absence of alamethicin is shown in panel C for the purpose of comparison Autoradiographs in C and C′ were exposed for 3 days, whereas those in panels A and B were exposed for 1 day

the channel-forming ionophore, alamethicin, to permit equilibration of reaction ingredients. Compared to control sarcolemmal vesicles, vesicles pretreated with alamethicin had greater than a 3-fold increase in^{32}P incorporation into the 15 kDa protein; the remaining bands were not significantly different from untreated controls (Fig. 2C vs. 2C′) [27]. Since alamethicin optimized ^{32}P incorporation, it was employed to phosphorylate sarcolemmal vesicles for [^{3}H]nitrendipine binding studies.

[^{3}H]Nitrendipine binding to phosphorylated and dephosphorylated sarcolemmal vesicles

Since alamethicin was employed in these studies to render the sarcolemmal vesicles permeable, it was necessary to determine whether alamethicin affected [^{3}H]nitrendipine binding. Treatment of vesicles with 1.0 mg alamethicin/mg sarcolemmal vesicle protein – an amount that has been shown to be optimal for permeabilizing the membrane to ATP and inorganic cations (Ref. 19, unpublished observations) – had no significant effect on either K_d or B_{max} for [^{3}H]nitrendipine (Table I). These data confirm previous findings that alamethicin is an efficient yet relatively innocuous agent for permeabilizing sarcolemmal vesicles [19].

A trivial explanation for the antagonism between dihydropyridines and isoproterenol would be that the latter binds to the 1,4-dihydropyridine receptor but does not produce calcium antagonism. Experiments were therefore carried out to test

TABLE I

EFFECT OF ALAMETHICIN TREATMENT ON [^{3}H]NITRENDIPINE BINDING TO CANINE SARCOLEMMAL VESICLES

[^{3}H]nitrendipine binding was performed as indicated in Methods and Materials K_d and B_{max} values are the mean ± S E and were determined using the LIGAND program of Munsen and Rodbard [26] n s, not significantly different from control values

	K_d (nM)	B_{max} (fmol/mg protein)
Control	0.096 ± 0 031	359 ± 78
+ alamethicin (1 mg/ml protein)	0.10 ± 0 05 n s	318 ± 106 n s

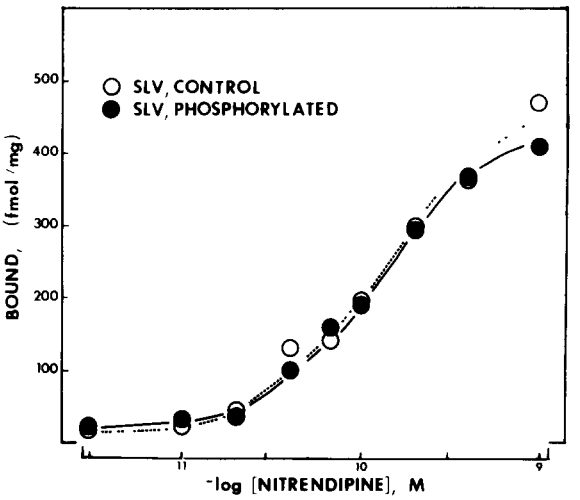

Fig. 3 Binding isotherm for [^{3}H]nitrendipine in control and phosphorylated cardiac sarcolemmal vesicles (SLV) Phosphorylation of sarcolemmal vesicles and binding measurements were carried out as described in Methods and Materials Each point represents the mean of triplicate determinations

whether or not isoproterenol might compete directly with [^{3}H]nitrendipine at the 1,4-dihydropyridine-binding site. The results shown in Table II indicate that a high concentration of isoproterenol (1.0 μM) had no significant effect upon binding.

Isotherms for [^{3}H]nitrendipine binding to either control or phosphorylated sarcolemmal vesicles are shown in Fig. 3. No significant differences in binding parameters were apparent between phosphorylated vesicles and their respective controls. Absolute values describing [^{3}H]nitrendipine binding in either control, phosphorylated or dephosphorylated sarcolemmal vesicles are shown in Table III. It is evident from these data that K_d or B_{max} values for this ligand in ventricular sarcolemmal vesicles are not measurably altered by extremes in phosphorylation state.

TABLE II

EFFECT OF ISOPROTERENOL ON [^{3}H]NITRENDIPINE BINDING TO CANINE SARCOLEMMAL VESICLES

[^{3}H]nitrendipine (NTD) binding to control sarcolemmal vesicles with alamethicin (1 0 mg/mg protein) was determined as indicated in Methods and Materials Data are presented as the mean ± S.E of triplicate determinations n s., not significantly different from the appropriate control values determined in the absence of isoproterenol (ISO)

[^{3}H]NTD (nM)	Bound (fmol/mg protein)	
	Control	+ 1 0 μM ISO
0.1	166 ± 24	141 ± 9 n s
1 0	477 ± 36	414 ± 69 n.s

TABLE III

EFFECT OF PHOSPHORYLATION AND DEPHOSPHORYLATION ON [^{3}H]NITRENDIPINE BINDING TO CANINE SARCOLEMMAL VESICLES (SLV)

[^{3}H]nitrendipine binding was determined as described in the Methods and Materials K_d and B_{max} values were obtained from analysis of the equilibrium binding curves by nonlinear least-squares methods Data are the means ± S E n.s., not significantly different from control values

Phosphorylation state	K_d (nM)	B_{max} (fmol/mg protein)
I Control (phosphorylation medium minus cAMP and ATP)	0 19 ± 0 046	571 ± 99
Phosphorylated SLV	0 14 ± 0 027 n s	479 ± 62 n.s
II Control (dephosphorylation medium minus phosphatase)	0 25 ± 0 018	665 ± 383
Dephosphorylated SLV	0 29 ± 0 06 n s	654 ± 102 n s

Discussion

Cardiac contraction occurs when Ca^{2+} enters the cytosol through the slow calcium channel (extracellular source) and releases calcium from the terminal cisternae of sarcoplasmic reticulum (intracellular source) [28]. During diastole, Ca^{2+} is removed from the cell by the Na^{+}-Ca^{2+} antiporter and a Ca^{2+}-ATPase [29]; intracellular stores are maintained by resequestering Ca^{2+} in the sarcoplasmic reticulum [30]. Although the Ca^{2+}-pumping activities of the sarcolemma and sarcoplasmic reticulum are mediated by phosphorylation reactions [29,30], it is not clear how phosphorylation regulates the electrically gated movement of Ca^{2+} through the slow channel.

The positive inotropic response associated with β-receptor agonists is thought to be primarily mediated by cAMP-dependent protein kinases which are known to phosphorylate several cardiomyocyte proteins [22,31]. While it is possible to relate this protein phosphorylation to acceleration of relaxation [29,30], it is more difficult to correlate increases in contractile force resulting from phoshorylation of specific proteins.

Lindemann et al. [5], Schneider and Sperelakis [32], and Vogel and Sperelakis [9] have proposed that a phosphorylation-dephosphorylation process occurring at the slow channel could control either the number or availability of functional Ca^{2+} channels. Phosphorylation of the 24 kDa protein located in the sarcolemmal vesicle and referred to as phospholamban by Manalan and Jones [27] and calciductin by Randli et al. [7] has been associated with stimulation of Ca^{2+} transport in both the sarcolemmal vesicle and sarcoplasmic reticulum. Besides myofibril and sarcoplasmic reticulum protein phosphorylation, Rinaldi et al. [7] have reported that phosphorylation of a 22–24 kDa protein in sarcolemmal vesicles coincides with increases in voltage-dependent Ca^{2+} uptake into sarcolemmal vesicles – an effect that could explain the positive inotropic effects of β-agonists. Although these authors concluded that they had demonstrated phosphorylation-dependent control of the slow Ca^{2+} channel in vitro, others have questioned the validity of this interpretation [33,34].

The present studies confirm that a cAMP-dependent phosphorylation of 24 kDa (12 kDa) and 15 kDa proteins occurs in sarcolemmal vesicles. These proteins are phosphorylated in a time-dependent manner and remain phosphorylated throughout the [^{3}H]nitrendipine-binding studies. In addition, binding can be studied in dephosphorylated sarcolemmal vesicles by pretreating vesicles with phosphatase prior to exposure to ligand. It is notable that dephosphorylation of the 15 kDa band proceeded slowly and was only 58% complete at 2 h. This may result from the location of this protein on the inside of sarcolemmal vesicles [27] and therefore relatively poor accessibility to the phosphatase.

Alamethicin was employed in these studies to allow consideration of both total phosphorylation and [^{3}H]nitrendipine-binding sites. The observation that disruption of the membrane barrier did not reveal additional binding sites, together with previous demonstrations that sarcolemmal vesicles are approx. 80% right-side-out suggests that [^{3}H]nitrendipine binds to the extracellular side of the plasma membrane. An alternate explanation would be that binding sites are on the interior of vesicles and that sarcolemmal membranes are relatively permeable to this drug. The present data do not allow us to distinguish between these two possibilities.

Our results indicate that cAMP-dependent phosphorylation of sarcolemmal membrane proteins does not alter the slow calcium channel in such a way that results in alterations in [^{3}H]nitrendipine binding to the high-affinity 1,4-dihydropyridine site; that is, phosphorylation did not significantly change either the affinity or number of measured binding sites. These data, therefore, do not support the thesis that catecholamine-induced antagonism of the negative inotropic effects of calcium channel blockers involves phosphorylation-dependent changes in binding parameters. Nevertheless, phosphorylation could result in a functional change in the slow calcium channel that is not detectable by the techniques employed here.

A shortcoming of this work is the inability to monitor the activity of the slow Ca^{2+} channel in sarcolemmal vesicles during manipulation of the phosphorylation state. Indeed, electrophysiological evidence suggests that calcium channels are nonfunctional in isolated membranes [35]. It is con-

ceivable that functional channels might be required to observe effects of phosphorylation upon calcium-antagonist binding; consequently, it would be desirable to carry out binding studies analogous to the present ones in intact cells such as cultured cardiomyocytes [36].

It should also be considered that Marsh et al. [36] have reported the presence of a second low-affinity binding site for [^{3}H]nitrendipine in cultured chick embryo heart cells. The K_d for binding at this site agreed well with the IC_{50} for inhibition of contractility; consequently, one might conclude that this low-affinity site may actually be the pharmacological receptor for nitrendipine. However, this observation notwithstanding, others have observed a high degree of correlation between pharmacological effects and [^{3}H]nitrendipine binding to the high-affinity site in this same system [37]. The cause for this discrepancy is not evident; nevertheless, these observations make it clear that further studies on the effects of phosphorylation state on nitrendipine binding in intact cardiomyocytes are warranted.

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