Dissociation of Phospholamban Regulation of Cardiac Sarcoplasmic Reticulum Ca\(^{2+}\)ATPase by Quercetin

(Received for publication, April 1, 1996, and in revised form, May 29, 1996)

Edward McKenna, Jeffrey S. Smith, Kathleen E. Coll, Elaine K. Mazack, Ernest J. Mayer, Joanne Antanavage, Richard T. Wiedmann, and Robert G. Johnson, Jr.

Merck Research Laboratories, Department of Pharmacology, WP44-B124, West Point, Pennsylvania 19486

Quercetin had a biphasic effect on Ca\(^{2+}\) uptake and calcium-stimulated ATP hydrolysis in isolated cardiac sarcoplasmic reticulum (SR). Stimulation of Ca\(^{2+}\)ATPase was observed at low quercetin concentrations (<25 \(\mu\)M) followed by inhibition at higher concentrations. The effects were dependent upon the SR protein concentration, the MgATP concentration, and intact phospholamban regulation of cardiac Ca\(^{2+}\)ATPase. Only the inhibitory effects at higher quercetin concentrations were observed in skeletal muscle SR which lacks phospholamban and in cardiac SR treated to remove phospholamban regulation. Stimulation was additive with monoclonal antibody 1D11 (directed against phospholamban) at submaximal antibody concentrations; however, the maximal antibody and quercetin stimulation were identical. Quercetin increased the calcium sensitivity of the Ca\(^{2+}\)ATPase like that observed with phosphorylation of phospholamban or treatment with monoclonal antibody 1D11. In addition, low concentrations of quercetin increased the steady-state formation of phosphoenzyme from ATP or \(P_i\), but higher quercetin decreased phosphoenzyme levels. Quercetin, even under stimulatory conditions, was a competitive inhibitor of ATP, but appears to relieve the Ca\(^{2+}\)ATPase from phospholamban inhibition, thereby, producing an activation. The subsequent inhibitory action of higher quercetin concentrations results from competition of quercetin with the nucleotide binding site of the Ca\(^{2+}\)ATPase. The data suggest that quercetin interacts with the nucleotide binding site to mask phospholamban’s inhibition of the SR Ca\(^{2+}\)ATPase and suggests that phospholamban may interact at or near the nucleotide binding site.

In cardiac and skeletal muscle, the sarcoplasmic reticulum (SR)\(^{1}\) Ca\(^{2+}\)ATPase is responsible for the reuptake of calcium into the SR to allow relaxation. Ca\(^{2+}\)ATPase transports two Ca\(^{2+}\) ions across the SR membrane against a large concentration gradient by hydrolytic coupling with one ATP molecule. Structural studies (Toyoshima et al., 1993; Stokes et al., 1994) suggest that Ca\(^{2+}\)ATPase molecules can exist in monomeric and oligomeric forms with enzymatic activity being regulated by its oligomeric state (Squier et al., 1988; Voss et al., 1991, 1994; Kutchai et al., 1994). Large aggregates of enzyme have low or no activity, and dissociated enzyme (possibly dimers) has high activity. Ca\(^{2+}\)ATPase exists in two chemically equivalent conformations that differ in their calcium affinity, \(E_1\) (high Ca\(^{2+}\) affinity) and \(E_2\) (low Ca\(^{2+}\) affinity). \(E_1\) binds Ca\(^{2+}\) cooperatively in a pH-dependent manner and \(E_2\) binds Ca\(^{2+}\) in a pH-independent and negatively cooperative manner (Nakamura and Tajima, 1995). ATP can modulate the intermolecular interactions to alter the relative populations of \(E_1\) and \(E_2\) (Scofano et al., 1979). There appears to be a single nucleotide binding site per Ca\(^{2+}\)ATPase (McIntosh and Boyer, 1983; Cable et al., 1985; Bishop et al., 1987; Lacapere and Guilain, 1993), which serves two functions, catalytic and regulatory. ATP concentrations above micromolar, which saturate the catalytic nucleotide binding site, serve a regulatory role to produce further increases in Ca\(^{2+}\)ATPase activity. Lastly, Ca\(^{2+}\) binding to \(E_1\) is accompanied by a protein conformational change which is an integral part of the cooperative binding mechanism and of enzyme activation by Ca\(^{2+}\). This conformational change is accelerated when millimolar MgATP is bound to the regulatory nucleotide binding site (Inesi et al., 1980; Stahl and Jencks, 1984; McIntosh and Davidson, 1984).

Phospholamban (PLB), an integral membrane protein present in cardiac SR but not in fast-twitch skeletal muscle SR, reduces the calcium sensitivity of the Ca\(^{2+}\)ATPase. Studies in PLB-deficient mice demonstrate the crucial role PLB plays in regulating basal contractile parameters and the heart’s responsiveness to hormonal activators (Luo et al., 1994). The phosphorylation domain and the nucleotide/hinge domain of the Ca\(^{2+}\)ATPase, and the cytoplasmic N terminus of PLB have been shown to be required for a PLB-regulated Ca\(^{2+}\)ATPase complex (Toyofuku et al., 1992, 1993, 1994). A recent model postulates that PLB inhibits the Ca\(^{2+}\)ATPase by aggregating it into a kinetically unfavorable associated state (Voss et al., 1994) through electrostatically controlled protein-protein interactions. Co-expression studies (Toyofuku et al., 1994), however, indicate that both charged and hydrophobic residues between amino acids 2 and 18 in PLB are essential for interaction with the Ca\(^{2+}\)ATPase. Relief of PLB inhibition can be achieved in one of several ways, viz. phosphorylation of PLB by protein kinases (Tada et al., 1974; Kranias, 1985), certain monoclonal antibodies directed against the N terminus of PLB (Cantilina et al., 1993; Mayer et al., 1996), mild trypsinization of PLB to remove its hydrophilic N terminus (Lu et al., 1993), nonsolubilizing concentrations of \(C_{12}E_8\) (Lu and Kirchberger, 1994), solubilization with deoxycholate and reconstitution of cardiac SR (Kim et al., 1990), treatment with heparin and related compounds (Xu and Kirchberger, 1989) or charged detergents (Chiesi and Schwaller, 1989), and the polyphenol tannin (Chiesi and Schwaller, 1994). These treatments all invoke the disruption of the interaction between PLB and the Ca\(^{2+}\)ATPase, presumably by direct effects on PLB, its lipid environment, or alterations in electrostatic protein-protein interactions.

---

\(^1\)The abbreviations used are: SR, sarcoplasmic reticulum; C\(_{12}\)E\(_{8}\), polyoxyethylene 8 lauryl ether; MOPS, 3-\(N\)-morpholino)propanesulfonic acid; PLB, phospholamban; Q, quercetin; Mes, 2-\(N\)-morpholinoethanesulfonic acid; mAb, monoclonal antibody.

\(^2\)To whom correspondence should be addressed. Tel.: 215-652-2128; Fax: 215-652-1658; E-mail: Edward_mckenna@merck.com.

\(^3\)The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 15 U.S.C. Section 1734 solely to indicate this fact.
teractions. No treatment that directly interacts with the Ca\(^{2+}\) ATPase to relieve it from PLB inhibition has been reported.

Quercetin (Q), a bioflavonoid, acts as an inhibitor of numerous enzymes involved in energy conversion reactions (Racker, 1986), phosphodiesterase (Kuppasamy and Das, 1992), and numerous protein kinases (Vlahos et al., 1994). Furthermore, it is an inhibitor of skeletal muscle Ca\(^{2+}\) ATPase (Shoshan and MacLennan, 1981; Fischer et al., 1987) with an IC\(_{50}\) of 12 \(\mu\)M in \(^{40}\)Ca\(^{2+}\) uptake experiments. Quercetin competitively inhibits ATP binding to the Ca\(^{2+}\) ATPase at low ATP concentrations (Shoshan and MacLennan, 1981) and has an affinity for the E-Ca\(_2\) (fully ligated with respect to calcium at the exterior high affinity calcium sites, unligated with respect to ATP) conformational state that is approximately 10-fold greater than for other conformational states in the hydrolytic cycle (Fischer et al., 1987). However, more detailed study of quercetin's effects on the partial reactions of the Ca\(^{2+}\) ATPase (Shoshan and MacLennan, 1981) indicate that the major effect of quercetin may not lie in its ability to inhibit nucleotide binding but possibly another step in the reaction sequence.

In this report, evidence suggesting that quercetin's biphasic effects result from its interaction with the nucleotide binding site on cardiac SR Ca\(^{2+}\) ATPase is presented. Stimulation appears to result from the disruption or masking of PLB's inhibitory effect on the Ca\(^{2+}\) ATPase, while the inhibitory effect at higher concentrations is like that observed in skeletal muscle SR.

**EXPERIMENTAL PROCEDURES**

**Materials**—\(^{32}\)P\(_{i}\)ATP and \(^{33}\)P\(_{i}\) were obtained from Amersham Corp. and \(^{40}\)CaCl\(_2\) from DuPont NEN. Quercetin and several common analogs and phosphoenol pyruvate were purchased from Sigma. Quercetin and similar compounds were dissolved in dimethyl sulfoxide or, in some experiments, in ethanol. Pyruvate kinase was from Boehringer Mannheim and A23187 from Calbiochem. Bovine pancreatic trypsin and soybean trypsin inhibitor were obtained from Worthington. Stimulatory anti-PLB mAb 1D11 was prepared as described by Mayer et al. (1996).

**Preparation of Sarcoplasmic Reticulum**—Canine cardiac sarcoplasmic reticulum was obtained as described previously (Mayer et al., 1996). Rabbit skeletal muscle sarcoplasmic reticulum was isolated from a discontinuous sucrose density gradient. Briefly, rabbit back and hindleg skeletal muscle was cleaned and minced. Muscle (100-g aliquots) were placed in 300 ml of buffer I (20 mM MOPS/KOH, pH 7.1, 0.3 M sucrose, and 1 mM phenylmethylsulfonyl fluoride) then homogenized in a Waring blender, two times, 30 s each. The homogenate was centrifuged at 2,600 \(\times\) g for 30 min and the supernatant was poured through cheesecloth. The pellet was resuspended in 200 ml of buffer I, and the homogenization and centrifugation steps were repeated. The two supernatants were combined and centrifuged at 10,000 \(\times\) g for 30 min.

The pellet was resuspended in 25 ml of buffer II (20 mM MOPS/KOH, pH 7.1, 0.3 M sucrose, and 0.6 M KCl) and incubated on ice for 1 h. Next, the sample was centrifuged at 165,000 \(\times\) g for 1 h, and the resulting pellet was resuspended in 4 ml of buffer III (20 mM MOPS/KOH, pH 7.1, 0.3 M sucrose, and 0.2 M KCl). The sample was applied to a discontinuous sucrose density gradient comprised of 10 ml of 45% sucrose, 15 ml of 30% sucrose, and 7 ml of 20% sucrose in buffer III. The gradient was centrifuged in a SW-28 rotor at 120,000 \(\times\) g for 4 h. Skeletal muscle SR, collected from the interface between 30 and 45% sucrose, was diluted in buffer III to fill a Ti45 centrifuge tube. Following centrifugation at 165,000 \(\times\) g for 1 h, the membrane pellet was resuspended in 3 ml of buffer III (per 100 g of muscle). Membranes were stored at \(-70^\circ\)C and protein determination was measured by the Pierce BCA protein assay with bovine serum albumin as a standard.

**Trypsin Treatment of Cardiac SR**—The procedure described by Lu et al. (1993) was adapted. Briefly, cardiac SR diluted to 1 mg/ml in buffer III was treated with 10 \(\mu\)g/ml trypsin for 5 min at 25 \(^\circ\)C. Control SR was prepared similarly except that trypsin was not added. The SR was diluted into 2 ml of ice-cold buffer III containing 0.12 mg/ml soybean trypsin inhibitor and centrifuged at 100,000 \(\times\) g for 30 min in a Beckman TLX tabletop ultracentrifuge. The resulting pellet was resuspended in buffer III to yield a 2 mg/ml protein concentration. Trypsin cleavage of PLB was verified by testing the effect of mAb 1D11 in the ATPase activity assay.

**Deoxycholate Solubilization and Reconstitution of Cardiac SR**—Cardiac SR was solubilized with 0.7 mg of deoxycholate/mg of SR protein as described by Kim et al. (1990). Deoxycholate was removed by addition of 0.5 g/ml Bio-Beads SM-2. The mixture was stirred for 1 h at room temperature. At the end of the incubation, the sample was recovered using a narrow bore pipette. Control cardiac SR was treated in a similar manner except deoxycholate was not added. As with the trypsinated samples, the loss of PLB-regulation was verified by testing for the effect of mAb 1D11 on ATPase activity.

**\(^{40}\)Ca\(^{2+}\) Uptake Measurement and ATPase Activity Microtiter Plate Assay**—These measurements were performed as described by Mayer et al. (1996).

**Phosphoenzyme Formation**—Phosphoenzyme was formed from \(^{32}\)P\(_{i}\)ATP or \(^{33}\)P\(_{i}\), using standard procedures. After preincubating 400
mu/ml cardiac SR in 150 mM KCl, 1 mM MgCl2, 5 mM NaN3, 0.5 mM EGTA, 20 mM imidazole, pH 7.0, with various concentrations of CaCl2 and quercetin for 20 min, formation of E1-P from 20 mM [33P]ATP was measured for 15 s at 0°C. Following preincubation for 20 min of 800 mu/ml cardiac SR in 40 mM Mes/Tris, pH 6.0, 10 mM MgCl2, 20% dimethyl sulfoxide, and varying amounts of quercetin, E2-P formation from 100 muM [33P]Pi was measured after 10 min at room temperature. The reaction was quenched with 0.25 M perchloric acid containing 4 mM Pi. Acid precipitated membranes were washed three times with 0.125 M perchloric acid, 4 mM Pi, and pelleted in a clinical centrifuge. Radiositive pellets were dissolved in 0.1 M NaOH, 2% Na2CO3, 1 mM Pi, and 2% sodium lauryl sulfate then counted in a scintillation counter using Ready Safe scintillation mixture.

RESULTS

Low micromolar concentrations of quercetin markedly increased calcium-dependent ATP hydrolysis in cardiac SR vesicles at submaximal free Ca2+. Stimulation of Ca2+ATPase is highly dependent on the ratio of quercetin to SR protein. In Fig. 1, at a cardiac SR protein concentration of 10 mu/ml and 0.5 mM MgATP (solid circles), maximal stimulation occurred at 3.16 muM quercetin which corresponds to 0.316 nmole of Q/mg of SR protein. If cardiac SR protein contains 1.12 pmol of Ca2+ATPase/mg of SR protein as determined by maximal phosphoenzyme formation (data not shown), then the maximal stimulatory ratio is about 282 nmol of Q/nmol of Ca2+ATPase.

A precipitous decline in Ca2+ATPase activity below the control rate occurred at quercetin concentrations above 10 muM. At 20 muM quercetin, cardiac SR Ca2+ATPase activity was about the same as control cardiac SR. At saturating free calcium concentrations above 1 muM, quercetin did not stimulate Ca2+ATPase activity, but the inhibitory effect at concentrations above 10 muM quercetin was still observed (data not shown). The half-maximal inhibitory concentration was about 20 muM quercetin at 10 mu/ml SR and 0.5 mM MgATP or 2.0 nmol of Q/mg of SR (1786 nmol of Q/nmol of Ca2+ATPase). The quercetin concentration response curve (not shown) on 45Ca2+ uptake into cardiac SR vesicles was studied in the presence of increasing concentrations of quercetin (up to 200 muM) at 10 mu/ml SR and 0.5 mM MgATP. The highest activity observed was about 3.16 muM quercetin which corresponds to 0.316 nmole of Q/mg of SR protein.

Paradoxical Quercetin Effects

Low levels of quercetin were found to stimulate Ca2+ATPase activity in cardiac SR vesicles. The effect was maximal at 3.16 muM quercetin, corresponding to 0.316 nmole of Q/mg of SR protein. This stimulation was highly dependent on the ratio of quercetin to SR protein. At 20 muM quercetin, cardiac SR Ca2+ATPase activity was about the same as control cardiac SR. At saturating free calcium concentrations above 1 muM, quercetin did not stimulate Ca2+ATPase activity, but the inhibitory effect at concentrations above 10 muM quercetin was still observed (data not shown). The half-maximal inhibitory concentration was about 20 muM quercetin at 10 mu/ml SR and 0.5 mM MgATP or 2.0 nmol of Q/mg of SR (1786 nmol of Q/nmol of Ca2+ATPase). The quercetin concentration response curve (not shown) on 45Ca2+ uptake into cardiac SR vesicles was studied in the presence of increasing concentrations of quercetin (up to 200 muM) at 10 mu/ml SR and 0.5 mM MgATP. The highest activity observed was about 3.16 muM quercetin which corresponds to 0.316 nmole of Q/mg of SR protein.

### TABLE I

| Name            | Substitution relative to flavone Ca2+ATPase activity | % of Me2SO control |
|-----------------|-------------------------------------------------------|--------------------|
| Fisetin         | 3-OH, 7-OH, 3'-OH, 4'-OH                              | 315                |
| Robinetin       | 3-OH, 3'-OH, 4'-OH, 5'-OH                             | 292                |
| Kaempferol      | 3-OH, 7-OH, 3'-OH, 4'-OH, 5'-OH                       | 285                |
| Quercetin       | 3-OH, 5-OH, 7-OH, 4'-OH, 5'-OH                        | 245                |
| Kaempferide     | 3-OH, 5-OH, 7-OH, 4'-Me                                | 228                |
| Rhamnetin       | 3-OH, 5-OH, 7-OCH3, 4'-OH, 5'-OH                       | 216                |
| Myricetin       | 3-OH, 5-OH, 7-OH, 3'-OH, 4'-OH, 5'-OH                  | 168                |
| 7-OHrhamnoside, 4'-OH, 5'-OH |                                                | 162                |
| Karanjin        | 3-O-Me, 7,8-Furan                                      | 159                |
|                 | 5-O-Me, 6-O-Me, 7-O-Me, 8-O-Me                         | 145                |
| Tectochrysin    | 3-OH, 7-O-Me                                          | 137                |
|                 | 5-OH, 6'-O-Me                                         | 129                |
| Ayancin Wollenweber | 3-O-Me, 5-OH, 7-O-Me, 4'-O-Me, 5'-OH                | 128                |
| Gardenia flavanoid | 5-OH, 6-O-Me, 7-O-Me, 5-OH, 3'-R, 4'-O-Me, 5'-R | 124                |
| Gard. B. Wollenweber | 5-OH, 6-O-Me, 7-O-Me, 5-O-Me, 4'-O-Me              | 123                |
|                 | 6-O-Me, 5'-O-Me                                       | 121                |
| Apigenin        | 5-OH, 7-OH, 4'-OH                                     | 120                |
|                 | 7-O-Me, 8-O-Me                                        | 120                |
| Chrysins        | 5-OH, 7-OH                                           | 120                |
|                 | 7-O(CH3)x5SCN                                         | 120                |
| Quercetagetin   | 3-OH, 5-OH, 6-OH, 7-OH, 4'-OH, 5'-OH                  | 119                |
| Arteninin       | 3-O-Me, 5-OH, 6-O-Me, 7-O-Me, 3'-O-Me, 4'-O-Me       | 115                |
| Roberin         | 3-O-Rhamnoside, 5-OH, 7-OHrhamnoside, 4'-OH           | 114                |
| Flavone         | None                                                  | 106                |
|                 | 3'-O-Me                                               | 105                |
|                 | 5-OH, 8-OH                                           | 95                 |
|                 | 3-COO-Me                                              | 93                 |
|                 | 7-OH, 8-OH                                           | 87                 |
|                 | 2'-O-Me, 5'-O-Me                                      | 82                 |
|                 | 5-OH, 6-Cl, 8-Cl                                      | 80                 |
|                 | 5-OH, 6-Cl                                           | 79                 |
| Acacetin        | 5-OH, 7-OH, 4'-O-Me                                   | 78                 |
|                 | 3-OH, 5-O-Me                                          | 75                 |
|                 | 2'-Cl, 4'-Cl                                          | 65                 |
|                 | 3'-O-Me, 4'-O-Me                                      | 62                 |
|                 | 5-OH, 2'-OH                                           | 62                 |
|                 | 4'-OH                                                 | 50                 |

*For gardenia flavanoid, R = mix of H and OMe.*
However, like cardiac SR, quercetin (skeletal muscle SR (Fig. 1, open circles) concentration of 12 mmol of Q/nmol of Ca^{2+} ATPase from the values of 10 μM quercetin and 3 μg/ml SR protein reported by Fischer et al. (1987). This disparate range of values exemplifies the complexity of the interaction of quercetin with the Ca^{2+} ATPase protein.

Fig. 2 shows the biphasic effect of protein concentration on the response of cardiac SR Ca^{2+} ATPase to a fixed quercetin concentration of 10 μM. Rates of calcium-stimulated ATP hydrolysis were normalized to matched solvent controls for each protein concentration and the relative rate versus the ratio of quercetin to SR protein are plotted. The free calcium concentration was 0.178 μM and the MgATP concentration was 0.5 mM. At low quercetin/protein ratios (<400 nmol of Q/mg of SR protein), a steep increase in Ca^{2+} ATPase activity was observed until an optimal ratio was achieved. At higher ratios, the stimulatory effect diminishes eventually leading to inhibition relative to the control Ca^{2+} ATPase activity. The optimal stimulatory ratio of quercetin to cardiac SR protein for this data was 400 nmol of Q/mg of SR protein.

Chemical structures of various flavanoids and their activity in modulating cardiac SR Ca^{2+} ATPase at 0.178 μM free Ca^{2+} are shown in Table I. mAb 1D11, typically, stimulated control Ca^{2+} ATPase activity around 300%. Each flavanoid (25 μM) was present 20 min prior to, and during the 30 min incubation with 0.5 mM MgATP. A wide range of Ca^{2+} ATPase activities were measured, some of these compounds tested at lower concentrations gave greater stimulation, e.g., 6 μM quercetin produced a 275% stimulation. This reversed dose dependence for stimulation of Ca^{2+} ATPase activity by quercetin suggests a similar biphasic dose-response profile, but with a different optimal stoichiometry of flavanoid to SR protein. A logical SAR for this group of flavanoids would require construction of complete dose-response curves for each compound to determine the optimal stoichiometry. The effects of some of these compounds at 25 μM were assayed on skeletal muscle SR Ca^{2+} ATPase under conditions identical to those used for cardiac SR, and the activities relative to the solvent control were fisetin (25%), robinetin (7%), quercetin (50%), myricetin (0%), and agenpin (73%), and quercetatin (5%).

The effect of saturating anti-PLB mAb 1D11 (Mayer et al., 1996) and an optimal stimulatory amount of quercetin on the apparent calcium sensitivity of cardiac SR Ca^{2+} ATPase activity were identical (Fig. 3A; Tables I and III). A leftward shift in the pCa curve indicating an apparent increased calcium sensitivity was observed. Furthermore, combining these treatments was not additive. Thus, quercetin appears to mimic mAb 1D11 in relieving the cardiac SR Ca^{2+} ATPase from the endogenous PLB inhibition. In another set of experiments, cardiac SR was solubilized with deoxycholate, and the detergent was removed with Bio-Beads to form intact vesicles that no longer exhibit PLB regulation (Kim et al., 1991). The loss of PLB inhibition was verified by the inability of the anti-PLB mAb 1D11 to stimulate the Ca^{2+} ATPase activity. The deoxycholate-treated SR Ca^{2+} ATPase activity versus pCa curve shows a leftward shift similar to that obtained following mAb 1D11 treatment. A submaximal quercetin dose was used to show an intermediate shift in apparent calcium sensitivity. Again, there was no additivity with quercetin and deoxycholate-treatment.

Stimulation by quercetin appears to require a PLB-regulated Ca^{2+} ATPase. Complete quercetin dose-response curves at a submaximal free calcium of 0.178 μM were constructed following two different procedures to eliminate PLB regulation (Fig. 4). First, cardiac SR was solubilized with deoxycholate and reconstituted as above. Deoxycholate-treatment stimulated Ca^{2+} ATPase activity almost 3-fold and abolished the stimulatory effect of quercetin. Quercetin’s inhibitory effect was not.

![Graph A](image1.png)

**Fig. 3.** A, calcium dependence of the effects of 25 μM quercetin on cardiac (●, ○) and mAb 1D11-treated (■, □) SR Ca^{2+} ATPase activity. Ca^{2+}-stimulated ATP hydrolysis was measured in triplicate at 37 °C over a range of free Ca^{2+} in the absence (●, ■) and presence of 25 μM quercetin (○, □). The curves were fitted to the equation $V = V_{max}/(1 + K_v/[Ca^{2+}]^{nH})$ where $n_H$ is the Hill coefficient and the fitted lines are shown. The results of the fit are summarized (Table II). The $V_{max}$ values are about three times higher than those shown below which were obtained at 25 °C. B, effects of 10 μM quercetin on cardiac (●, ○) and deoxycholate-solubilized (■, □) SR Ca^{2+} ATPase activity. Ca^{2+}-stimulated ATP hydrolysis was measured in triplicate over a range of free Ca^{2+} in the absence (●, ■) and presence of 10 μM quercetin (○, □). Curve fits were performed and the fitted lines are shown. The results of the fit are summarized in Table III.

vesicles was congruent with the curve shown in Fig. 1 except the protein concentration was 100 μg/ml and the MgATP concentration was 3.16 mM. The optimal stimulatory ratio of quercetin to SR protein was 223 nmol of Q/mg of SR protein.

Quercetin did not stimulate Ca^{2+} ATPase activity in rabbit skeletal muscle SR (Fig. 1, open circles) which lacks PLB. However, like cardiac SR, quercetin (>10 μM concentration) markedly inhibited the skeletal muscle SR Ca^{2+} ATPase as previously reported (Shoshan and MacLennan, 1981; Fischer et al., 1987). Half-maximal inhibition occurred at 40 μM for 5 μg/ml skeletal muscle SR which corresponds to a ratio of 8.0 nmol of Q/mg of SR protein. Using an estimate of 4.5 nmol of Ca^{2+} ATPase/mg of skeletal muscle SR (Shoshan and MacLennan, 1981; Bishop et al., 1987), then half-maximal inhibition of skeletal muscle SR Ca^{2+} ATPase occurred at a ratio of 1778 nmol of Q/nmol of Ca^{2+} ATPase, which is identical to the value calculated above for cardiac SR. A value of 89 nmol of Q/nmol of Ca^{2+} ATPase was calculated using a half-maximal inhibitory concentration of 12 μM quercetin and 30 μg/ml protein as reported by Shoshan and MacLennan (1981) and 740 nmol of Q/nmol of Ca^{2+} ATPase from the values of 10 μM quercetin and 3 μg/ml SR protein reported by Fischer et al. (1987).
altered. An alternative treatment using mild trypsination of cardiac SR (Lu et al., 1993) which results primarily in the loss of the cytoplasmic domain of PLB was used. Proteolysis of PLB was nearly complete, as treatment of trypsinized cardiac SR with the stimulatory anti-PLB mAb 1D11 produced only a trace amounts of intact PLB following Western blot analysis (data not shown). Again, quercetin no longer exerted a stimulatory effect, while its inhibitory effect was unchanged.

The ability of quercetin to mimic deregulation of PLB inhibition is best illustrated by the quercetin dose-response curves (Fig. 5) constructed in the presence of increasing amounts of mAb 1D11 ranging from none to a saturating amount of 10 μg/ml. Each curve peaks at the same quercetin concentration of 25 μM. Moreover, the maximal Ca\(^{2+}\)·ATPase activity approaches the same rate which is identical to the maximal mAb 1D11 effect. The additive effect of quercetin and mAb 1D11 at submaximal concentrations is clear-cut, but the mechanism of quercetin’s effect needed clarification.

A direct interaction of stimulatory amounts of quercetin and other flavanoids with PLB was checked. None of the compounds at 25 μM concentration altered the mAb 1D11 antibody binding in a competitive enzyme-linked immunosorbent assay (data not shown). The effect of short cytoplasmic PLB-derived peptides on quercetin-induced stimulation of cardiac SR Ca\(^{2+}\)·ATPase activity was assayed in the \(^{45}\)Ca\(^{2+}\) uptake assay (Fig. 6). None of the peptides at concentrations up to 100 μM affected the basal Ca\(^{2+}\)·ATPase activity. Neither the PLB 7–17 peptide nor the synthetic protein kinase A substrate, LR-RASLG (Kemptide), had any effect on quercetin’s stimulatory action. Synthetic PLB peptide 1–25 at 50 μM produced about a 40% reduction of the maximal quercetin stimulation, yet 5 μM PLB 1–25 completely abolished the maximal stimulation by mAb 1D11 or by protein kinase A catalyzed phosphorylation (data not shown). Furthermore, 1% polyvinyl pyrrolidone, a non-specific chelator of phenolic compounds, produced a similar 40% inhibition of quercetin-induced stimulation. Therefore, it does not appear that quercetin has a specific interaction with PLB.

Quercetin is well known to affect ATP-dependent enzymes (Racker, 1986) and is an inhibitor of skeletal muscle SR Ca\(^{2+}\)·ATPase (Shoshan and MacLennan, 1981). The MgATP dependence of cardiac SR Ca\(^{2+}\)·ATPase under conditions of quercetin stimulation was characterized and compared with the stimulation produced by mAb 1D11 (Fig. 7A). At low MgATP (5.62 μM), 10 μM quercetin had no apparent effect on Ca\(^{2+}\)·ATPase activity, and yet at saturating MgATP (500 μM), this same quercetin concentration rivaled the maximal mAb 1D11 response. The data are replotted in Fig. 7B and Table IV as an Eadie-Scatchard plot. The \(K_m\) for MgATP under control Ca\(^{2+}\)·ATPase assay conditions (1% Me2SO) is 6.7 μM, and the \(V_{max}\) is 58.5 nmol of P/mg/min. Addition of mAb 1D11 had no effect on the \(K_m\) for MgATP (6.9 μM), but the \(V_{max}\) was almost doubled to 111.9 nmol of P/mg/min. Thus, mAb 1D11 is a noncompetitive activator of cardiac SR Ca\(^{2+}\)·ATPase or, more accurately, PLB is a noncompetitive inhibitor of cardiac SR Ca\(^{2+}\)·ATPase. Quercetin likewise doubled the \(V_{max}\) to 121.8 nmol of P/mg/min, but it also significantly increased the \(K_m\) for MgATP to 31.3 μM. Even under optimal stimulating conditions, quercetin acts as a competitive inhibitor for MgATP activation of cardiac SR Ca\(^{2+}\)·ATPase. However, stimulation is observed because quercetin removes the inhibition by PLB.

MgATP dependence curves were performed in the presence of varying quercetin concentrations producing both stimulatory and inhibitory effects. A linear Dixon plot of apparent \(K_m\) versus each quercetin concentration was obtained (Fig. 8). A \(K_i\) estimate of 26.4 μM quercetin was determined. This value is in the same range as the optimal stimulatory quercetin concentration in the presence of saturating MgATP. Taken together these experiments suggest that quercetin interacts with the nucleotide binding site of Ca\(^{2+}\)·ATPase and this site is associated with the PLB binding site.

The effect of quercetin on cardiac SR Ca\(^{2+}\)·ATPase phosphoenzyme formation in the forward and reverse modes was studied. \(E_1^{-}\)P is formed in the presence of Ca\(^{2+}\) and ATP, while \(E_2^{-}\)P is formed from P\(_i\) in the absence of ATP. At a submaximal Ca\(^{2+}\) concentration of 0.1 μM (Fig. 9), low stoichiometries of quercetin to Ca\(^{2+}\)·ATPase (1–100 nmol of quercetin/mg of SR protein) moderately increased the steady-state formation of \(E_1^{-}\)P (20–25% increase) and \(E_2^{-}\)P (40% increase). In contrast, mAb 1D11 produces a 100% increase of steady-state \(E_1^{-}\)P level and an identical 40% increase in \(E_2^{-}\)P at this same calcium concentration. The discrepancy in the stimulation of \(E_1^{-}\)P formation observed with quercetin and mAb 1D11 may be explained by quercetin’s interaction with the nucleotide binding site at the low ATP concentration (20 μM) used in the \(E_1^{-}\)P phosphoenzyme experiments. At high stoichiometries of quercetin to Ca\(^{2+}\)·ATPase, formation of phosphoenzyme is greatly diminished in both modes. Presumably, 2

E. McKenna, J. S. Smith, K. E. Coll, E. K. Mazack, E. J. Mayer, J. Antanavage, R. T. Wiedmann, and R. G. Johnson, Jr., unpublished data.
this indicates that quercetin inhibits formation of the phosphorylated enzyme intermediate by competing with ATP at the nucleotide binding site.

**DISCUSSION**

**Quercetin’s Interaction with Ca\(^{2+}\) ATPase**—The results show that the enzymatic activity of cardiac SR Ca\(^{2+}\) ATPase, but not of skeletal muscle SR Ca\(^{2+}\) ATPase, is stimulated at submicromolar Ca\(^{2+}\) by low concentrations of quercetin. Stimulation is not observed at saturating Ca\(^{2+}\) (>1 mM). The stimulatory action is attenuated at higher quercetin concentrations followed by inhibition of enzyme activity. The stimulatory effect requires intact PLB regulation of the cardiac Ca\(^{2+}\) ATPase, as following treatments that disrupt PLB regulation only the inhibitory range of quercetin activity remains. Furthermore, the maximal stimulation is identical to that observed when PLB regulation is removed and quercetin stimulation is additive with submaximal amounts of mAb 1D11. The inhibitory concentration range of quercetin in cardiac SR is the same as in skeletal muscle SR (which lacks PLB) and in cardiac SR treated to remove PLB regulation. The inhibitory effects of quercetin on skeletal muscle SR Ca\(^{2+}\) ATPase have been well characterized (Shoshan and MacLennan, 1981; Fischer et al., 1987). Quercetin inhibits the binding of ATP and ADP to Ca\(^{2+}\) ATPase, but not the binding of calcium (Shoshan and MacLennan, 1981). Indeed, quercetin had preferential affinity for the enzyme fully ligated with respect to exterior high affinity Ca\(^{2+}\) binding sites and unligated with respect to ATP (Fischer et al., 1987). However, as noted by Shoshan and MacLennan (1981) and shown in the phosphoenzyme experiments in Fig. 9, quercetin may stabilize a conformational intermediate that cannot energize the release of Ca\(^{2+}\) into the SR lumen nor donate Pi back to ADP. Indeed, quercetin did not reduce the steady-state level of phosphoenzyme over the drug concentration range that inhibits ATP hydrolysis, implying that quercetin can bind to phosphorylated forms of the enzyme that have an unoccupied regulatory nucleotide binding site. Thus, in cardiac SR, quercetin appears to obfuscate the inhibitory effects of PLB, thereby producing an apparent stimulation (i.e. quercetin is not a direct activator), prior to producing inhibitory effects as described for skeletal muscle SR Ca\(^{2+}\) ATPase.

The effects of quercetin and other bioflavanoids were variable and appear to be dependent on the assay conditions. There is a complex relationship between the Ca\(^{2+}\) ATPase, PLB, and substrates which must be considered when interpreting the results. Quercetin has a biphasic protein-dependence in cardiac SR (Fig. 2), which may be explained by a necessary stoichiometry of quercetin to Ca\(^{2+}\) ATPase molecules to disrupt PLB
regulation. This stoichiometry is a complex function of quercetin and ATP concentrations due to competitive binding at the nucleotide site.

Both the stimulatory and inhibitory effects of quercetin appear to result from direct interaction with the nucleotide binding site of Ca\(^{2+}\) ATPase. No evidence for a direct quercetin-PLB interaction or quercetin antioxidative activity on the SR phospholipids was found. Quercetin acts as a powerful antioxidant against lipid peroxidation when lipid bilayers are exposed to aqueous oxygen radicals (Terao et al., 1994). It is most likely to be localized near the surface of phospholipid bilayers. No alterations in \(^{45}\)Ca uptake rates in control and quercetin-treated cardiac SR were found (data not shown) when measured in the absence of oxygen and the presence of ascorbate. The ATP dependence data (Fig. 7, A and B) demonstrate quercetin’s competitive inhibition for nucleotide binding in cardiac SR, whereas PLB acts as a noncompetitive inhibitor.

The data clearly shows that stimulation by quercetin requires a PLB-regulated Ca\(^{2+}\) ATPase. Under conditions where PLB regulation has been disrupted, e.g. deoxycholate treatment, limited trypsinization, in micromolar Ca\(^{2+}\), or in saturating anti-PLB mAb 1D11, only inhibition by quercetin is observed. The maximal stimulation elicited by quercetin corresponds to the complete disruption of PLB regulation. Moreover, quercetin stimulation is additive with submaximal amounts of anti-PLB mAb 1D11 (Fig. 5) and the \(V_{\text{max}}(\text{MgATP})\) using mAb 1D11 or quercetin were the same (Fig. 7B). Quercetin interacts with the nucleotide binding site of Ca\(^{2+}\) ATPase and removes the inhibitory effect of PLB. This results in an increase in the number of active Ca\(^{2+}\) ATPase units. Thus, the anti-PLB or “phospholamban inhibitor” effect of quercetin causes the stimulation of Ca\(^{2+}\) ATPase activity.

Phospholamban’s Interaction with Ca\(^{2+}\) ATPase: Recruitment Hypothesis—PLB regulation involves the ability of the Ca\(^{2+}\) ATPase to be activated by Ca\(^{2+}\) and ATP without altering the affinity of substrate binding. Calcium and nucleotide binding to cardiac SR Ca\(^{2+}\) ATPase has an affinity and cooperativity identical to skeletal muscle SR (Cantilina et al., 1993). Recent work (Voss et al., 1994) suggests that PLB regulation of the cardiac Ca\(^{2+}\) ATPase is related to critical changes in protein

![FIG. 7.](image)

**A**. MgATP dependence of the effect of 10 \(\mu\)g/ml mAb 1D11 and 25 \(\mu\)M quercetin on Ca\(^{2+}\)-stimulated ATP hydrolysis. Ca\(^{2+}\) ATPase activity was measured in triplicate as described in Fig. 1 in the presence of an ATP-regenerating system. The rates of Ca\(^{2+}\)-stimulated ATP hydrolysis in control (●) and mAb 1D11-treated (●) and quercetin-treated (■) cardiac SR were determined in the presence of 3.16 \(\mu\)M to 3.16 mM MgATP (iATP). B. Eadie-Scatchard plot of \(V(\text{ATP})\) versus \(V\). The data shown in A were transformed to yield the \(V(\text{ATP})\) versus \(V\) plot. The x intercept is the \(V_{\text{max}}\) and the slope = \(1/K_m\). The results from a linear regression fit are summarized in Table IV.

![FIG. 8.](image)

**FIG. 8.** Dixon plot for a competitive inhibitor of MgATP activation of Ca\(^{2+}\) ATPase activity. The effect of quercetin concentrations ranging from 5 to 100 \(\mu\)M on the MgATP concentration dependence of Ca\(^{2+}\) ATPase was measured. For each quercetin concentration, Eadie-Scatchard plots were constructed, and the \(K_m(\text{ATP})\) was determined. The x intercept equals \(-K_m(\text{quercetin})\) and the y intercept is the \(K_m(\text{MgATP})\) for control cardiac SR. The linear regression fit of the data is shown yielding a \(K_m(\text{quercetin})\) of 26 \(\mu\)M.

| Treatment          | \(V_{\text{max}}(\text{MgATP})\) nmol Pi/mg/min | \(K_m(\text{MgATP})\) M |
|--------------------|-----------------------------------------------|------------------------|
| Control            | 58.2 ± 8.0                                   | 6.57 ± 0.43            |
| 10 \(\mu\)g/ml mAb 1D11 | 111.7 ± 20.0                               | 6.84 ± 0.66            |
| 25 \(\mu\)M quercetin | 121.9 ± 15.1                               | 31.15 ± 1.96           |

TABLE IV

Effect of mAb 1D11 and quercetin on the \(V_{\text{max}}(\text{MgATP})\) and the \(K_m(\text{MgATP})\).
dynamics and protein-protein interactions. According to their hypothesis, PLB inhibits Ca\textsuperscript{2+} ATPase by aggregating it into large oligomeric complexes, and phosphorylation of PLB activates by dissociating these large aggregates. Alternatively, mAb 1D11 or saturating micromolar Ca\textsuperscript{2+} produced the same effect. We would like to extend their hypothesis to state that the PLB-aggregated Ca\textsuperscript{2+} ATPase molecules are enzymatically inactive. Disruption of PLB-aggregated Ca\textsuperscript{2+} ATPase allows enzymatic activity to occur. In effect, disruption of PLB-regulation increases the availability of active Ca\textsuperscript{2+} ATPase units, i.e., recruitment of more pumps from a PLB-aggregated reserve.

The recruitment hypothesis is supported by experimental evidence here as well as in numerous publications. For years, researchers have argued that the shift in apparent calcium sensitivity of cardiac SR Ca\textsuperscript{2+} ATPase observed following PLB phosphorylation or treatment with anti-PLB mAb was solely a kinetic effect as described by Cantilina et al. (1993). An alternative explanation is that unphosphorylated PLB aggregates and inactivates a population of Ca\textsuperscript{2+} ATPase molecules. PLB-phosphorylation or anti-PLB mAb treatment releases the inactive pool of Ca\textsuperscript{2+} ATPase causing an increase in the number of active pump units resulting in a leftward shift in the pCa curve (Fig. 3, A and B). Quercetin produced a concentration-dependent increase in the steady-state level of phosphoenzyme intermediate (Fig. 9), i.e., an increase in the number of active pump units. Similarly, Cantilina et al. (1993) (see their Fig. 7) measured the formation of phosphoenzyme intermediate in the absence and presence of anti-PLB mAb and at varied Ca\textsuperscript{2+} concentration. Their contention was that disruption of PLB-regulation mimicked the effect of micromolar Ca\textsuperscript{2+} increasing the rate of phosphoenzyme formation and hence produced a kinetic effect similar to other reports. However, they failed to recognize the importance of an increase in steady-state E–P level at each subsaturating Ca\textsuperscript{2+} which can be due only to an increase in number of Ca\textsuperscript{2+} ATPase units. The steady-state phosphoenzyme level reflects the availability of Ca\textsuperscript{2+} ATPase molecules to undergo catalysis and is dependent upon the amount of Ca\textsuperscript{2+} bound to the Ca\textsuperscript{2+} ATPase. Thus, more Ca\textsuperscript{2+} ATPase units turning over per unit of time produces an apparent increase in the rate of phosphoenzyme formation.

The MgATP dependence of the cardiac SR Ca\textsuperscript{2+} ATPase shown in Fig. 7, A and B, clearly demonstrates an increase in the number of active Ca\textsuperscript{2+} ATPase units upon addition of anti-PLB mAb 1D11. There was no effect on the \(K_m\) for nucleotide, but the \(V_{max}\) which reflects the number of active Ca\textsuperscript{2+} ATPase units, increased. Similar results were obtained following partial trypptic digest of PLB in cardiac SR vesicles (Lu et al., 1993) (Fig. 4). The observation that quercetin also increases the \(V_{max}\) suggests that it is increasing the number of active pumps by disrupting PLB-Ca\textsuperscript{2+} ATPase interaction. Furthermore, non-solubilizing concentrations of C\textsubscript{12}E\textsubscript{8} (Lu and Kirchberger, 1994) or C\textsubscript{12}E\textsubscript{9} produce equivalent effects on \(V_{max}\). Low concentrations of C\textsubscript{12}E\textsubscript{9} partially disaggregate Ca\textsuperscript{2+} ATPase in cardiac SR providing a physical correlate for the functional effects (Shi et al., 1996). Hence, we would expect quercetin to have similar effects on Ca\textsuperscript{2+} ATPase aggregation.

Recent studies with transgenic PLB-deficient mice and comparisons with their heterozygous, wild-type, and PLB overexpressing relatives add supporting evidence to the recruitment hypothesis. The SR Ca\textsuperscript{2+} ATPase of PLB-deficient mice has a high apparent Ca\textsuperscript{2+} sensitivity compared to the wild-type counterpart, while cardiac SR from the heterozygous mice (40% of wild-type) have an intermediate Ca\textsuperscript{2+} sensitivity (Luo et al., 1994). In transgenic mice overexpressing PLB relative to wild-type mice, there is an additional rightward shift in the pCa curve (Kadambi et al., 1996), EC\textsubscript{50} = 0.27 \(\mu\)M (wild-type) versus 0.48 \(\mu\)M (overexpressed). The rightward shift in the pCa curves with increasing PLB expression suggests a progressive inactivation of Ca\textsuperscript{2+} ATPase units. It follows that the role of PLB in heart muscle is to maintain a reserve pool of Ca\textsuperscript{2+} ATPase units for periods of increased contractile activity and not merely to inhibit the Ca\textsuperscript{2+} pumping activity.

REFERENCES
Bishop, J. E., Al-Shawi, M. K., and Inesi, G. (1987) J. Biol. Chem. 262, 4658–4663
Cable, M. B., Feher, J. J., and Briggs, F. N. (1985) Biochemistry 24, 5612–5619
Cantilina, T., Sagara, Y., Inesi, G., and Jones, L. R. (1993) J. Biol. Chem. 268, 17018–17025
Chiesi, M., and Schwallier, R. (1989) FEBS Lett. 244, 241–244
Chiesi, M., and Schwallier, R. (1994) Biochem. Biophys. Res. Commun. 202, 1668–1673
Fischer, T. H., Campbell, K. P., and White, G. C. (1987) Biochemistry 26, 8024–8039
Inesi, G., Kurzmark, M., Coan, C., and Lewis, D. E. (1980) J. Biol. Chem. 255, 3025–3031
Kadambi, V. J., Ponniah, S., Harrer, J. M., Hoit, B. D., Dorn, G. W., Walsh, R. A., and Kranias, E. G. (1996) J. Clin. Invest. 99, 533–537
Kim, H. W., Steenaart, N. A. E., Ferguson, D. G., and Kranias, E. G. (1990) J. Biol. Chem. 265, 1702–1709
Kranias, E. G. (1985) Biochim. Biophys. Acta 844, 193–199
Kuppasany, U. R., and Das, N. P. (1992) Biochim. Biophys. Acta 1173, 401–409
Lacapere, J.-J., and Guillain, F (1993) Eur. J. Biochem. 211, 117–126
Lu, Y.-Z., and Kirchberger, M. A. (1994) Biochemistry 33, 5056–5062
Lu, Y.-Z., Xu, Z.-C., and Kirchberger, M. A. (1993) Biochemistry 32, 3105–3110
Luo, W., Grupp, I. L., Harrer, J., Ponniah, S., Grupp, G., Duffy, J. D., Doetschman, T., and Kranias, E. G. (1994) Circ. Res. 75, 401–409
Mayer, E. M., McKenna, E., Garsky, V. M., Burke, C. J., Mach, H., Middaugh, C. R., Sarlana, S., Smith, J. S., and Johnson, R. G. (1996) J. Biol. Chem. 271, 1669–1677
McIntosh, D. B., and Boyer, P. (1983) Biochemistry 22, 12867–12874
McIntosh, D. G., and Davidson, G. A. (1984) Biochemistry 23, 1959–1965
Nakamura, J., and Tajima, G. (1993) J. Biol. Chem. 70, 17350–17354

2 E. McKenna, J. S. Smith, K. E. Coll, E. K. Mazack, E. J. Mayer, J. Antanavage, R. T. Wiedmann, and R. G. Johnson, Jr., unpublished results.
Paradoxical Quercetin Effects

Racker, E. (1986) Prog. Clin. Biol. Res. 213, 257–271
Scofano, H. M., Vieyra, A., and de Meis, L. (1979) J. Biol. Chem. 254, 10227–10231
Shi, Y., Karon, B. S., and Thomas, D. D. (1996) Biophys. J. 70, A283
Shoshan, V., and MacLennan, D. H. (1981) J. Biol. Chem. 256, 887–892
Squier, T. C., Hughes, S. E., and Thomas, D. D. (1988) J. Biol. Chem. 263, 9162–9170
Stahl, and Jencks, W. P. (1984) Biochemistry 23, 5389–5392
Stokes, D. L., Taylor, W. R., and Green, N. M. (1994) FEBS Lett. 346, 32–38
Tada, M., Kirchberger, M. A., Repke, D. I., and Katz, A. M. (1974) J. Biol. Chem. 249, 6174–6180
Terao, J., Piskula, M., and Yao, Q. (1994) Arch. Biochem. Biophys. 308, 278–284
Toyofuku, T., Kurzydlowski, K., Lytton, J., and MacLennan, D. H. (1992) J. Biol. Chem. 267, 14490–14496
Toyofuku, T., Kurzydlowski, K., Tada, M., and MacLennan, D. H. (1993) J. Biol. Chem. 268, 2809–2815
Toyofuku, T., Kurzydlowski, K., Tada, M., and MacLennan, D. H. (1994) J. Biol. Chem. 269, 3088–3094
Toyoshima, C., Sasabe, H., and Stokes, D. L. (1993) Nature 362, 469–471
Vlahos, C. J., Kriaucinaius, T. D., Gleason, P. E., Jones, J. A., Eble, J. N., Salvas, D., Falcone, J. F., and Hirsch, K. S. (1993) J. Cell. Biochem. 52, 404–413
Voss, J., Hassey, D., Birmachu, W., and Thomas, D. D. (1991) Biochemistry 30, 7488–7506
Voss, J., Jones, L. R., and Thomas, D. D. (1994) Biophys. J. 67, 190–196
Xu, Z.-C., and Kirchberger, M. A. (1989) J. Biol. Chem. 264, 16644–16651