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Poly(dA-dT)·Poly(dA-dT) Two-Pathway Proton Exchange Mechanism. Effect of General and Specific Base Catalysis on Deuteration Rates†

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ABSTRACT: The deuteration rates of the poly(dA-dT)·poly(dA-dT) amino and imino protons have been measured with stopped-flow spectrophotometry as a function of general and specific base catalyst concentration. Two proton exchange classes are found with time constants differing by a factor of 10 (4 and 0.4 s^{-1}). The slower class represents the exchange of the adenine amino protons whereas the proton of the faster class has been assigned to the thymine imino proton. The exchange rates of these two classes of protons are independent of general and specific base catalyst concentration. This very characteristic behavior demonstrates that in our experimental conditions the exchange rates of the imino and amino protons in poly(dA-dT)·poly(dA-dT) are limited by two different conformational fluctuations. We present a three-state exchange mechanism accounting for our experimental results.

The opening–closing reaction in double-stranded nucleic acids, resulting from the breaking of the base pair hydrogen bonds, is an essential component of the dynamic structure of these molecules (Englander & Kallenbach, 1983). Because the hydrogen exchange between water protons and protons involved in hydrogen bonding requires a prior breaking of the hydrogen bonds, the measurement of the hydrogen exchange kinetics can yield important information concerning both the mechanism of this opening reaction and the nature of the open state.

Most, if not all, hydrogen exchange measurements were analyzed according to the simple, two-step reaction mechanism

$$C \rightleftarrows O \rightarrow \text{exchange} \qquad (1)$$

In this scheme C stands for the closed native state with hydrogen-bonded base pairs, whereas O stands for the open state characterized by ruptured hydrogen bonds. Although the exact nature of the open state is unknown, it is generally assumed that in this state the base pairs are fully exposed to solvent, allowing the base protons to exchange freely with water protons at a rate comparable to the one obtained with mononucleotides. This reaction scheme, albeit reasonable, has not yet received large experimental support. Its wide use results mainly from its simplicity in allowing the easy extraction of crude information (concerning the opening reaction) from the exchange rate.

The only experimental evidence in favor of reaction scheme 1 was given by Mandal et al. (1979). Using stopped-flow spectrophotometry, these authors measured the deuteration rate of the poly(rA)·poly(rU) amino and imino protons as a function of general catalyst concentration. They found that the fast exchange rate of the imino proton is independent of catalyst concentration and is thus limited by the opening rate of the base pairs whereas the exchange rate of the amino protons levels off with increasing catalyst concentration at a value equal to the imino proton exchange rate. Such a characteristic exchange pattern in response to high catalysis is predicted by the two-step exchange model (eq 1). Recently, these results have been questioned by Leroy et al. (1985). Deducing the poly(rA)·poly(rU) imino proton exchange rate from nuclear magnetic resonance line broadening, they observed a strong dependence of the imino proton band width with catalyst concentration. This result, in contrast with the one of Mandal et al. (1979), seems to indicate that in the absence of catalyst the imino proton is not limited by the opening rate of the base pairs. The imino proton exhibits a similar behavior in poly(rI)·poly(rC). However, despite this conflicting result, Leroy et al. agreed with the exchange mechanism (eq 1).

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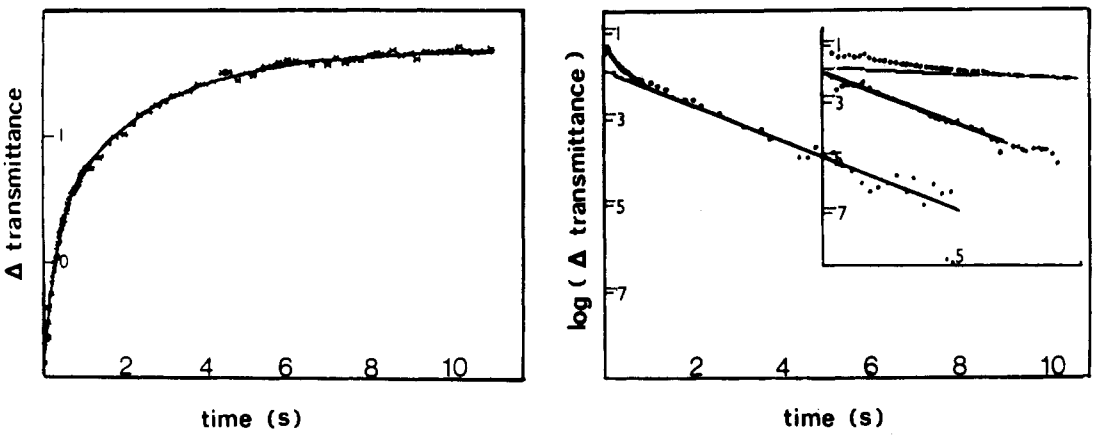

FIGURE 1: (Left) Typical kinetic trace for the hydrogen–deuterium exchange of poly(dA-dT)·poly(dA-dT) at 20 °C observed by transmittance detection at 285 nm after stopped-flow mixing. The dots represent the experimental data and the solid line represents the computed fit. The buffer was 10^{-1} M sodium chloride and 10^{-2} M sodium phosphate, pH 6.5. (Right) Plot of log $(T_t - T)$ against time, where T_t is the signal value at time t and T is the final asymptotic signal value; the slope of the straight line obtained by linear regression gives the rate constant of the slow phase. (Inset) Expanded time scale to follow the early time data; deducting the slow phase from the experimental curve gives the time course of the fast phase; the corresponding points can be fitted by a straight line, whose slope is equal to the rate constant of the fast phase. The amplitudes of the fast and slow phases are obtained by extrapolating to time $t = 0$ the corresponding straight lines.

To test the generality of mechanism 1, we have used stopped-flow spectrophotometry to study the deuteration rate of the imino and amino protons in poly(dA-dT)·poly(dA-dT) as a function of pH and catalyst concentration. Both rates, though different, are independent of pH and catalyst concentration. Altogether, these results indicate that exchange mechanism 1 is not operative as such in poly(dA-dT)·poly(dA-dT) and has to be modified. We present a three-state exchange mechanism that accounts for our experimental data.

Experimental Procedures

Materials. Poly(dA-dT)·poly(dA-dT) was obtained from P-L Biochemicals. For concentration determinations the extinction coefficient 6800 M^{-1} cm^{-1} at 260 nm was used. The polynucleotide was treated with phenol, precipitated with ethanol, and then extensively dialyzed against the buffer. Prior to use, the poly(dA-dT)·poly(dA-dT) solution was heated to 80 °C for 10 min and then slowly cooled to room temperature. The size of our poly(dA-dT)·poly(dA-dT) sample was measured by electrophoresis on 1% agarose gel, and it was found to be larger than 25 000 base pairs.

Sodium chloride and sodium phosphate were from Merck, and tris(hydroxymethyl)aminomethane (Tris) was from Boehringer.

Deuterium oxide (99.5%) was purchased from CEA (Saclay, France). The pD values of the deuterium oxide solutions were determined by the approximation of Glasoe and Long (1960): $pD = pH_{meter} + 0.4$. All pH values were measured at 20 °C with a Tacussel Minisis pH meter.

Methods. (*1*) *Stopped-Flow Kinetics.* To measure the rate of hydrogen exchange in poly(dA-dT)·poly(dA-dT), we followed the deuterium-labeling rate with a Durrum D 110 (Dionex) stopped-flow instrument interfaced with a Hewlett-Packard 9826 computer. The progress curves were obtained as a time-dependent change in transmittance at 285 nm. As the change in transmittance is small (<10%), this is proportional to the optical density. One hundred points per kinetic measurement were stored under computer control. These data were then represented as a semilogorithmic plot and analyzed for amplitude and rate constant with a linear least-squares routine.

The mixing dead time of the instrument was found to be approximately 3 ms, and the slide width was 0.4 mm. The ultraviolet light source was a 75-W xenon arc lamp, and the

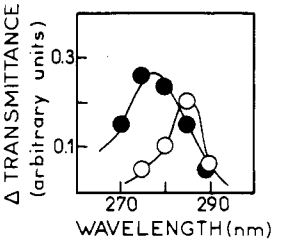

FIGURE 2: Kinetic difference spectra for the deuteration of poly(dA-dT)·poly(dA-dT): (●) slow phase; (○) fast phase. These spectra indicate kinetically determined transmittance differences in arbitrary units upon mixing samples from H_2O into D_2O in the stopped-flow instrument. The buffer was 10^{-1} M sodium chloride and 10^{-2} M Tris, pH 7.1.

optical path length in the observation cell was 2 cm. All experiments were performed with a 1 to 1 mixing ratio. The final concentration of the polymer after being mixed with D_2O buffer in the stopped-flow instrument was always 3.9×10^{-4} M. The temperature of the flow system (drive syringes, mixing and observation chamber) was maintained within ±0.2 °C of the desired temperature.

(*2*) *Circular Dichroism Spectra.* Before all hydrogen exchange measurements on poly(dA-dT)·poly(dA-dT), we routinely recorded a dichroism spectra with a Jobin Yvon Mark IV dichrograph in order to check the conformation of the polymer. The spectra were similar to those previously published for poly(dA-dT)·poly(dA-dT) with an alternating B conformation (Vorlickova et al., 1983; Vorlickova & Kypr, 1985). The increase of Tris and phosphate buffer concentrations did not affect the spectra.

Results

Recorded at 285 nm as transmittance, a typical stopped-flow kinetic trace corresponding to the deuteration rate of poly(dA-dT)·poly(dA-dT) is presented in Figure 1. On the same figure is also shown the corresponding semilogarithmic plot. As can be seen from this curve, the deuteration rate of poly(dA-dT)·poly(dA-dT) is a two-exponential process with rate constants equal to 0.45 s^{-1} and 4 s^{-1} for the slow phase and fast phase, respectively. In Figure 2 the corresponding amplitudes are plotted as a function of wavelength. Similar results were obtained by Nakanishi et al. (1984).

Because of their different chemical exchange pathways, the amino protons of free adenine are sensitive around neutrality

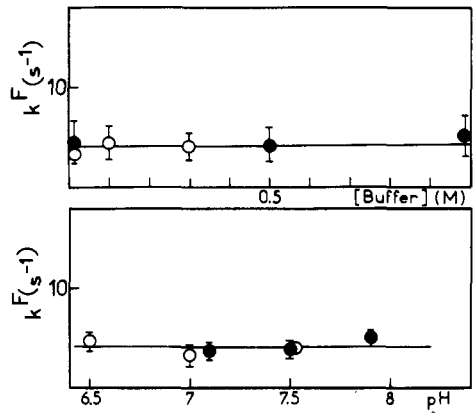

FIGURE 3: (Top) Rate constant of the fast phase as a function of total buffer concentration at 20 °C: (●) Tris buffer, 10^{-1} M sodium chloride, pH 7.1; (○) sodium phosphate buffer, 10^{-1} M sodium chloride, pH 6.5. (Bottom) Rate constant of the fast phase as a function of pH at 20 °C: (●) 10^{-1} M sodium chloride, 5×10^{-1} M Tris; (○) 10^{-1} M sodium chloride, 10^{-1} M sodium phosphate. The data points are the averaged value obtained from five to ten determinations, and the vertical lines represent twice the corresponding standard deviation.

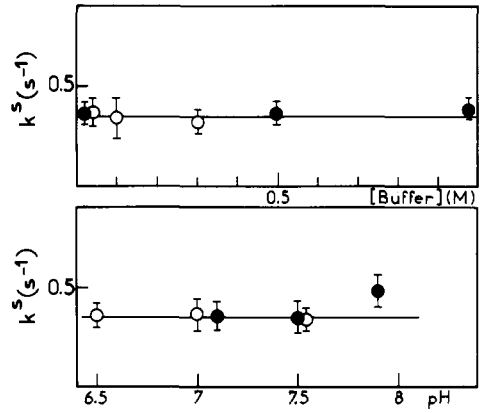

FIGURE 4: (Top) Rate constant of the slow phase as a function of total buffer concentration at 20 °C. The meaning of the symbols is as in Figure 3. (Bottom) Rate constant of the slow phase as function of pH at 20 °C. The meaning of the symbols is as in Figure 3. The data points and the corresponding vertical lines are obtained as in Figure 3.

to an apparent general acid catalysis (Teitelbaum & Englander, 1975a; Cross et al., 1975) whereas the thymine imino proton by comparison with the uracil imino proton can be confidently expected to be sensitive to both specific and general base catalysis (Mandal et al., 1979; Fritzsche et al., 1981). Thus we have studied the effect of specific catalysis and general catalysis using phosphate and Tris buffers. Figures 3 and 4 show that the fast and slow exchange rates are independent of phosphate and Tris buffer concentration at pH values where the acidic form of the buffer is predominant. The measured exchange rates, as a function of increasing pH in the presence of 0.5 M Tris and 0.1 M phosphate, are also presented in Figures 3 and 4; within the limit of experimental error the deuteration rates in poly(dA-dT)·poly(dA-dT) do not exhibit either any general base or any specific base catalysis.

Discussion

As a starting point for the discussion we have to assign the protons of the two exchanging kinetic classes. Nakanishi et al. (1984) have measured the poly(dA-dT)·poly(dA-dT) deuteration rate constants of the fast and slow classes as a function of temperature. Comparing their data with the hydrogen–tritium exchange rates of the poly(dA-dT)·poly(dA-dT) imino and amino protons measured by Teitelbaum and Englander (1975a), they found that the imino and amino proton exchange rates measured by tritium labeling fall on the Arrhenius plots of the fast and slow deuteration rates, respectively. This allowed these authors to assign confidently the exchanging protons of the fast and slow phases, respectively, to the thymine imino proton and adenine amino protons. Because the deuteration rate constants of the fast and slow phases determined in our work are in the same range as those obtained by Nakanishi et al. (1984), it is clear that the same identification holds in our case.

As already stated, our data indicate that the imino and amino poly(dA-dT)·poly(dA-dT) protons are not affected by specific or general catalysis. To fully appreciate the meaning of these results, it is necessary to evaluate the acceleration factors expected for freely solvent exposed thymine imino and adenine amino protons in our most extreme experimental conditions. Let us examine first the thymine imino protons. Thymine is the same base as uracil except for the thymine methyl group, and therefore it can be assumed that the exchange mechanism of the thymine imino proton is identical with that of the uracil imino proton, which has been extensively studied by NMR spectroscopy (Mandal et al., 1979; Fritzsche et al., 1981). In the present pH range, the limiting step of the proton exchange process is the direct imino proton removal by either OH^- (specific base catalysis) or the base form of a buffer (general base catalysis). The corresponding rate is given by

$$k_{EX}^{Im} = k_D[B]\frac{10^{\Delta pK}}{1 + 10^{\Delta pK}} \quad (2)$$

where k_D is the diffusion-limited encounter rate constant and [B] is the catalyst concentration (either OH^- or the buffer base form). The factor $10^{\Delta pK}/(1 + 10^{\Delta pK})$, where ΔpK is catalyst pK − H exchange group pK, reflects the rate constant dependence upon the relative acidities between the imino group and the base. In the case of OH^- it reduces to unity whereas for phosphate and Tris buffers these factors are respectively 1 order and 2 orders of magnitude smaller owing to their less favorable pKs. The previous formula indicates that an increase of the Tris concentration from 10^{-2} to 1 M should accelerate the thymine imino proton rate corresponding to buffer catalysis by a factor of 100; on the other hand, an acceleration factor of 30 is expected when phosphate concentration varies from 10^{-2} to 0.3 M. Similarly, a pH increase from 6.5 to 7.9 should raise the imino OH^- catalyzed rate by a factor of 25.

The chemistry of the exchange of adenine amino protons is also well understood (Teitelbaum & Englander, 1975a,b; Cross et al., 1975; Mc Connell, 1974). The limiting step exchange rate is again the removal of an adenine amino proton by OH^- (specific base catalysis) or by the buffer base form (general base catalysis). But to facilitate the exchange (the amino group pK is very unfavorable), the exchanging adenine has first to be protonated at the position N_1; this changes the amino proton pK to a value ($pK \sim 9$) where the amino protons are withdrawn at a diffusion-limited rate. The corresponding exchange rate is

$$k_{EX}^{Am} = [H^+]K_pk_D\frac{10^{\Delta pK}}{1 + 10^{\Delta pK}}[B] \quad (3)$$

K_p is the equilibrium protonation constant at the N_1 position ($K_p \sim 4.3$) and the meanings of all the other terms are as in expression 2. If [B] stands for $[OH^-]$, it can be noticed that the rate equation (eq 3) involves the product $[H^+][OH^-]$ and thus predicts a pH-independent rate for the adenine amino

proton. However, expression 3 shows that the adenine amino protons are affected by general base catalysis, which in fact appears as an apparent general acid catalysis because the product $[H^+][B]$ is proportional to the acidic form of the buffer. The acceleration factor calculated from the rate equation (eq 3) when Tris concentration increases from 10^{-2} to 1 M is 100 whereas the acceleration factor for a phosphate concentration increase from 10^{-2} to 0.3 M is around 30.

Despite our selected experimental conditions that lead, as we have just shown, to important acceleration of the exchange rate of freely solvent exposed nucleotide, we could not find evidence in the case of the polymer for any increase of the deuteration rate for both the fast and slow classes. These would correspond respectively to the exchange of the thymine imino proton and of adenine amino protons. Let us now examine the consequences of these experimental data at the dynamic structure level of poly(dA-dT)·poly(dA-dT). According to the two-step reaction proposed by Teitelbaum and Englander (1975a,b)

$$C \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} O \xrightarrow{k_{ch}} \text{exchange} \qquad (4)$$

$$K_{eq} = k_{op}/k_{cl}$$

the flux of exchanging protons per time unit is given by

$$k_{EX} = k_{op}k_{ch}/(k_{cl} + k_{ch}) \qquad (5)$$

where k_{op} and k_{cl} represent the opening and closing rates of the base pairs and K_{eq} is the opening equilibrium constant. k_{ch} is the chemical rate constant whose values can be estimated from expression 2 or 3 on the assumption that the base pairs in the open form are equivalent to freely solvent exposed nucleotides. As we shall see, this hypothesis can be questioned in the case of adenine amino protons of poly(dA-dT)·poly(dA-dT). A limiting regime of special interest in relation with our data is obtained when $k_{ch} \gg k_{cl}$. In this case expression 4 reduces to

$$k_{EX} = k_{op} \qquad (6)$$

The exchange of the proton is opening-limited: each time the hydrogen bonds of a base pair are severed, exchange of the protons takes place. This exchange regime can be tested easily by experiment as it is expected to be independent of all factors increasing k_{ch}, as long as these factors do not affect the parameters governing the conformational dynamic rate constants, k_{op} and k_{cl} in our model.

Our experimental results (rate of imino proton unaffected by general and specific base catalysis, rate of amino protons unaffected by general base catalysis) are indicative of an opening-limited regime, providing that the catalyst we are using can form an encounter complex with the exchanging proton during the transient open state. In the case of the thymine imino proton it is clear that at least the OH^- ion must be able to form an encounter complex in the open state; if not, then this proton would not exchange at all. Hence, as the exchange rate of this proton is not accelerated between pH 6.5 and pH 7.9, we are lead to the unescapable conclusion that the poly(dA-dT)·poly(dA-dT) imino protons are opening-limited at 20 °C.

Concerning the amino protons, two extreme situations can account for the independence of the exchange rate upon general catalyst concentration. First, the adenine base in the open state does not swing out into the solvent and instead remains staked with the exchanging protons buried inside the double helix. This would prevent the formation of an encounter complex between the catalyst and the exchanging amino group. Second, the exchange rate of the protons is simply limited by some fluctuations of the poly(dA-dT)·poly(dA-dT) conformation. We favor the second explanation because it is difficult to visualize a transient intermediate open form where the amino protons must be accessible to some extend to the OH^- ion (otherwise they would not exchange) but are completely shielded from both phosphate and Tris buffer base forms. In the case of an opening-limited exchange of the adenine amino protons, the measured exchange rates of these protons are equal to the rate of some conformational fluctuation that is necessary to bring the adenine in contact with the solvent. As this rate is 10 times smaller than the corresponding fluctuation rate leading to the exchange of the thymine imino proton, we must conclude that in poly(dA-dT)·poly(dA-dT) the imino and amino protons are exchanging via two different pathways, each characterized by its own fluctuation mode. This situation can be most simply summarized by the three-state model

$$\text{exchange} \leftarrow \text{OUT(thymine)} \rightleftarrows \text{native} \rightleftarrows \text{OUT(adenine)} \rightarrow \text{exchange} \qquad (7)$$

According to this reaction scheme the protons, which cannot exchange from the native state where the base pairs are hydrogen-bonded, have to wait for some prior structural fluctuations that differ for the imino and amino protons. These lead either to the OUT(thymine) state or the OUT(adenine) state where the thymine imino proton and the adenine amino protons, respectively, are exposed to solvent and can therefore exchange. We would like to emphasize that the exact nature of the states OUT(thymine) and OUT(adenine) are unknown and thus the term "OUT" should not be taken too literally.

In conclusion, it is worthwhile to compare our data obtained with poly(dA-dT)·poly(dA-dT), the conformation of which is an alternating right-handed helix [for a review, see Vorlickova & Kypr (1985)], with the proton exchange data of a double-stranded nucleic acid that has a different conformation in solution. By far the most widely studied double-stranded nucleic acid is poly(rA)·poly(rU), which adopts an A conformation in solution. This polymer has been examined by tritium labeling, by stopped-flow spectrophotometry, and by NMR. Although there exists a sharp disagreement between the opening-limited value of the uracil imino proton exchange rate determined on one hand by stopped-flow spectrophotometry (Mandal et al., 1979) and on the other by NMR (Leroy et al., 1985), both groups adopt the classical two-step mechanism (eq 4) to analyze their data. This reaction scheme has also been used in the case of poly(rG)·poly(rC) and poly(rI)·poly(rC) (Teitelbaum & Englander, 1975b; Leroy et al., 1975), which also adopt in solution an A conformation. Thus, for all the members of the A conformation family so far studied, the two-step mechanism (eq 4) can account for experimental data. This is not the case of Z-DNA. Using tritium labeling, we have previously measured the proton exchange rate in poly(dG-dC)·poly(dG-dC) Z-DNA (Ramstein & Leng, 1980; Ramstein et al., 1985). Besides the overall exchange rates being orders of magnitude slower than in all the other polynucleotides studied so far [the slowest protons in poly(dA-dT)·poly(dA-dT) are around 3 orders of magnitude faster than the slowest protons in poly(dG-dC)·poly(dG-dC)], we have shown that the classical two-step mechanism (eq 4) is not operative in the case of Z-DNA. In contrast, the Z-DNA exchange could be most simply analyzed with a three-state exchange scheme similar to the one proposed here in the case of poly(dA-dT)·poly(dA-dT). It is tempting to speculate on the possible existence of a relationship between this exchange mechanism and the characteristic alternated confor-

mation of poly(dA-dT)·poly(dA-dT) and of poly(dG-dC)·poly(dG-dC) Z-DNA.

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Registry No. Poly(dA-dT)·poly(dA-dT), 26966-61-0; H_2, 1333-74-0.

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Reconstitution of the Voltage-Sensitive Calcium Channel Purified from Skeletal Muscle Transverse Tubules†

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ABSTRACT: The purified calcium antagonist receptor of the voltage-sensitive calcium channel from skeletal muscle transverse tubule membrane consists of three subunits: α with M_r 135 000, β with M_r 50 000, and γ with M_r 33 000. Purified receptor preparations were incorporated into phosphatidylcholine (PC) vesicles by addition of PC in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and removal of detergent by molecular seive chromatography. Forty-five percent of the α, β, and γ polypeptides and the [^{3}H]dihydropyridine/receptor complex were recovered in association with PC vesicles. The rate of dissociation of the purified and reconstituted dihydropyridine/receptor complex was identical with that in T-tubule membranes, and allosteric modulation by verapamil and diltiazem was retained. The reconstituted calcium antagonist receptor, when occupied by the calcium channel activator BAY K 8644, mediated specific $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ transport into the reconstituted vesicles. $^{45}Ca^{2+}$ influx was blocked by the organic calcium antagonists PN200-110 ($K_{0.5}$ = 0.2 μM), D600 ($K_{0.5}$ = 1.0 μM), and verapamil ($K_{0.5}$ = 1.5 μM) and by inorganic calcium channel antagonists ($La^{3+} > Cd^{2+} > Ni^{2+} > Mg^{2+}$) as in intact T-tubules. A close quantitative correlation was observed between the presence of the α, β, and γ subunits of the calcium antagonist receptor and the ability to mediate $^{45}Ca^{2+}$ or $^{133}Ba^{2+}$ flux into reconstituted vesicles. Comparison of the number of reconstituted calcium antagonist receptors and functional channels supports the conclusion that only a few percent of the purified calcium antagonist receptor polypeptides are capable of mediating calcium transport as previously demonstrated for calcium antagonist receptors in intact T-tubules.

Voltage-sensitive calcium channels mediate an increase in cytosolic calcium in response to depolarization and play an important role in excitation–contraction coupling in cardiac and smooth muscle (Hagiwara & Byerly, 1981; Tsien, 1983). The major class of calcium channel in muscle is modulated by dihydropyridine calcium agonists and antagonists (Janis & Triggle, 1983; Schramm & Towart, 1985). These compounds increase and decrease cellular calcium currents by stabilizing long open and closed states of an ensemble of individual calcium channels (Kokubun & Reuter, 1984; Hess et al., 1984). Their binding is subject to allosteric modulation by structurally diverse calcium antagonists that bind to two additional lower affinity sites and either enhance (e.g., diltiazem and other benzothiazepines) or inhibit (e.g., verapamil and other phenylalkylamines) dihydropyridine binding (Schramm & Towart, 1985; Janis & Triggle, 1984).

Skeletal muscle fibers have large voltage-sensitive calcium currents that are restricted to the T-tubule system and are modulated by dihydropyridine calcium channel agonists and

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