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The Pharmacology of Ryanodine and Related Compounds[a]

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I. Introduction

It has been 25 years since the seminal review by Jenden and Fairhurst, *The Pharmacology of Ryanodine* (Jenden and Fairhurst, 1969), appeared in this Journal. During this time, we have learned a great deal about ryanodine and related compounds, and about ryanodine receptor (RyR)[c] calcium release channels, the family of proteins that bind and are modified by these ligands. The interest in the pharmacology of the RyRs has increased as these proteins have been shown to serve as intracellular Ca^{2+} release channels in a variety of tissue and cell types (Sutko and Airey, 1996). Moreover, as discussed in this review, several important questions remain concerning the role of RyR-mediated Ca^{2+} release events in different cell types, and the channel properties of different RyR isoforms. Resolution of these questions will require, at least in part, the use of pharmacological agents that modify the activity of the RyRs in both channel property- and isoform-specific ways. Recent data suggest it will be possible to develop such agents. Compounds related to ryanodine, termed ryanoids, exhibit agonist- and antagonist-like actions on RyR channels and, if not provided by nature, have the potential to be made RyR isoform-specific by chemical engineering. In addition, new agents that affect RyR channels, many of which are chemically unrelated to ryanodine, are being identified at a rapid rate.

In this article, we focus on the present state of RyR pharmacology and consider the aspects of RyR function that require the development of RyR isoform-specific RyR channel modifiers. Ryanodine and related compounds will receive much of our attention, as they remain the prototypic and most potent modulators of the RyR family of proteins. We will also limit our consideration to vertebrate RyRs. As noted below, the ryanoids are commercially important pesticides, and the effects of these agents on insects have been discussed previously (Crosby, 1971; Pessah, 1990; Jefferies and Casida, 1994).

[c] Abbreviations: RyR, ryanodine receptor; DDT, dichlorodiphenyltrichloroethane; SR, sarcoplasmic reticulum; RER, rough endoplasmic reticulum; DHPR, dihydropyridine receptor; IP_3, inositol triphosphate; ATP, adenosine triphosphate; ISM, interconvertible site model; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DSM, distinct site model; K_D, dissociation constant; EC_{50}, median effective concentration; NMR, nuclear magnetic resonance; CoMFA, comparative molecular field analysis; FKBP, FK 506 binding protein; cADPR, cyclic adenosine diphosphate ribose.

II. Ryanodine: Historical Aspects

Ryanodine is a natural product found in members of the genus *Ryania*, which grow as shrubs or slender trees in several tropical locations in Central and South America, including Trinidad, and the Amazon basin. In addi-

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tion, a closely related compound, ryanodol (**2**) has been isolated from the wood of *Persea indica* (Gonzalez-Coloma et al., 1993). It has long been appreciated that wood from trees in the genus *Ryania* contains toxic components. Crude extracts were used by local natives to poison arrowheads (Crosby, 1971). In the 1940s before the widespread use of synthetic chlorinated hydrocarbon and organic phosphate insecticides, E.F. Rogers, K. Folkers and colleagues at Merck and Co., and members of the Department of Entomology at Rutgers University conducted a cooperative survey of plant materials for new insecticides. This work revealed that extracts of the stem and roots of *Ryania speciosa* Vahl had promising insecticidal activity. Rogers et al. (1948) purified a compound from stem wood that they designated ryanodine, which had 700 times the insecticidal potency of the starting material.

Powdered *Ryania* wood was marketed initially as an insecticide by the Penick Corporation, but became less profitable as synthetic compounds, such as DDT and parathion, came into wide usage during the 1950s and 1960s. The detrimental environmental impact of the latter insecticides and the increased desire for organically grown produce led to the reintroduction of *Ryania* wood as an insecticide by Tom and Lorraine Harding of Progressive Agri-Systems (DeVault, 1983). A mixture of *Ryania* wood, rotenone, and pyrethrum has proven to be particularly effective. Ryanodine retains its value as an insecticide for several reasons. First, it is as effective as DDT or parathion against crop-damaging insects, such as the Codling moth (apples) and the European corn borer. Second, it is biodegradable, with a relatively short half-life, permitting it be applied close to harvest and avoiding the environmental concerns associated with persistence. Third, in several cases, the selectivity of ryanodine's insecticidal actions is fortuitous in that crop-damaging insects are affected, while beneficial insects are spared. These properties led ryanodine, rotenone, and pyrethrum to be identified as acceptable alternative insecticides by Rachel Carson in her book, "Silent Spring" (Carson, 1962).

The availability of purified ryanodine suitable for biomedical research largely has been due to the efforts of a few individuals. These include Dr. E.F. Rogers (Merck Sharp and Dohme Research Laboratory, West Point, PA), Dr. Ronald Harmetz (Penick Corporation), and Lorraine and Tom Harding (Progressive Agri-Systems). A debt of gratitude is owed to these individuals, who have selflessly worked to keep ryanodine preparations suitable for research available to the biomedical community during times when it was not an economically viable product.

Finally, ryanodine is also of historical significance because its properties and biological actions have permitted the identification and molecular characterization of a family of intracellular Ca^{2+} release channels, now commonly termed the ryanodine receptors.

III. The Ryanodine Receptor Ca^{2+} Release Channels

The RyRs are a family of Ca^{2+} channels that participate in the release of Ca^{2+} from intracellular stores. To date, the most notable of these stores are the sarcoplasmic reticulum (SR) in muscle and the rough endoplasmic reticulum (RER) in nonmuscle cells (Sutko and Airey, 1996). In addition, recent data suggest a greater variety in the way cells use these proteins, as RyRs have been reported to release Ca^{2+} from stores associated with the nucleus (Gerasimenko et al., 1995) and to be used to sense extracellular Ca^{2+} in osteocytes (Zaidi et al., 1992, 1995). In this section, we consider RyR distribution, structure, and function to the extent necessary to describe specific issues that would benefit from the development of new pharmacological agents that affect RyR function. Readers desiring more information about these topics are referred to several recent reviews of the molecular and functional aspects of the RyRs (Williams, 1992; McPherson and Campbell, 1993; Ogawa, 1994; Coronado et al., 1994; Meissner, 1994; Sutko and Airey, 1996).

A. Molecular identity

RyRs were initially observed in skeletal muscle, where they were visualized in electron micrographs as large electron-dense masses situated along the face of the SR terminal cisternae, which is closely apposed to transverse tubule membranes to form a structure known as the triad junction (Franzini-Armstrong, 1970, 1972). Based on their appearance, the RyRs were termed triad junctional foot proteins (Franzini-Armstrong, 1970, 1972, 1975). The RyRs gained their present name after they were found to be the proteins that bind [^{3}H]ryanodine (Pessah et al., 1985; Inui et al., 1987, 1988; Campbell et al., 1987; Lai et al., 1988), an agent known from earlier studies to alter SR calcium release events (Fairhurst, 1974; Fairhurst and Hasselbach, 1970).

The RyR family is proving to be extensive. Three genes encoding different RyR isoforms have been described in mammalian tissues. They have been cloned and sequenced from skeletal muscle (*ryr1*), heart (*ryr2*), and brain (*ryr3*) (Takeshima et al., 1989; Marks et al., 1989; Zorzato et al., 1990; Nakai et al., 1990; Otsu et al., 1990; Giannini et al., 1992; Hakamata et al., 1992). Sequence homologs of the three mammalian RyR isoforms are expressed also in nonmammalian vertebrates, where they have been termed α (RyR1), β (RyR3) and cardiac (RyR2) (Airey et al., 1990, 1993c; Olivares et al., 1991; Murayama and Ogawa, 1992; Lai et al., 1992; Dutro et al., 1993; O'Brien et al., 1993; Ogawa, 1994; Oyamada et al., 1994; Jens et al., 1995; Conti et al., 1996; Ottini et al., 1996). In addition, two alternatively spliced variants of RyR1 and one variant of RyR2 have been identified (Nakai et al., 1990; Zorzato et al., 1994; Futatsugi et al., 1995). These splice variants involve

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deletions of five (Ala (3481)-Gln (3485)) (Zorzato et al., 1994; Futatsugi et al., 1995) and six (Val (3865)-Asn (3870)) (Futatsugi et al., 1995) amino acids within a region of the RyR1 molecule that contains several putative regulatory sites. These variants are expressed in both a tissue- and a developmental stage-specific manner, suggesting that they may have different functional properties and/or may be regulated in different ways (Futatsugi et al., 1995).

There also may be RyR isoforms that are distinct from the mammalian and nonmammalian vertebrate isoforms described in the preceding paragraph. For example, a ryanodine binding activity in mammalian liver exhibits distinctive properties (Shoshan-Barmatz, 1990; Shoshan-Barmatz et al., 1991; Bazotte et al., 1991; Lilly and Gollan, 1995). A RyR purified from lobster muscle requires much greater Ca^{2+} concentrations for channel activation than the vertebrate isoforms characterized to date (Olivares et al., 1993; Seok et al., 1992). The sequence of a RyR from *Drosophila* tissues is the most divergent identified to date (Hasan and Rosbach, 1992; Takeshima et al., 1994b), exhibiting $< 50\%$ homology with the vertebrate isoforms (Takeshima et al., 1994b).

A truncated version of the skeletal muscle RyR (RyR1), which is translated using an alternate start site within the RyR1 mRNA, has been found in mammalian brain (Takeshima et al., 1993). Although the functional properties of this protein have not yet been described, the protein consists of the C-terminal ~75 kDa of RyR1, a region thought to contain the Ca^{2+} channel domain. Consequently, this protein also may be a Ca^{2+} channel. As described below, two different approaches starting with the intact RyR have localized the high affinity ryanodine binding site to the region contained in the truncated RyR. However, cells expressing the truncated protein did not exhibit detectable [^{3}H]ryanodine binding activity (Takeshima et al., 1993). Therefore, it is unclear whether the C-terminal 75 kDa protein alone is sufficient to bind ryanodine.

The channel properties of the mammalian RyR1 and RyR3 isoforms, the αRyR and βRyR isoforms in chickens, frogs, and fish, and the RyR expressed in lobster muscle have been investigated in vitro (Seok et al., 1992; Bull and Marengo, 1993; Olivares et al., 1993; Percival et al., 1994; O'Brien et al., 1995). Significant differences in the gating and activation of the channels associated with these proteins have been described, indicating that the diversity of this family of channels underlies different roles for each member in intracellular Ca^{2+} signaling.

B. Distribution

The RyRs have broad phylogenetic and tissue distributions. Biochemical, molecular or pharmacological evidence for the presence of RyRs has been found in invertebrates (lobster, *Drosophila* and *C. elegans*), as well as vertebrates (mammals, birds, reptiles, fish, and amphibians) (Sutko and Airey, 1996). Some vertebrate cell types that express RyRs include neurons in both the central and peripheral nervous systems, smooth muscle, endothelial cells, adrenal chromaffin cells, hepatocytes, osteocytes, eggs, and pancreatic cells (Sutko and Airey, 1996). In many of these cells, the identity of the specific RyR isoforms expressed, the intracellular distributions of these proteins and the cellular responses elicited by RyR-mediated Ca^{2+} release events are not known and represent important topics for future study.

Other complexities involving RyR-mediated Ca^{2+} release signals are that the RyRs expressed within a cell are localized to different sites where they may have different functions, or where more than one RyR isoform is co-expressed. For example, RyRs expressed in skeletal muscle cells may be have both extrajunctional and junctional distributions (Dulhunty et al., 1992). The RyR expressed in cardiac muscle (RyR2) is localized to different regions of the SR. One region involves junctions between the SR and the surface or t-tubule membranes, while a second region, termed corbular SR, consists of SR membranes that form terminal, junctional-like structures in the interior of the cell that do not form junctions with another membrane (Jewett et al., 1973; Jorgensen et al., 1993; Carl et al., 1995). Localization to specialized regions within the cell is likely to confer different properties on the Ca^{2+} release events mediated by each population of RyRs. Freeze-fracture studies of toadfish swim bladder muscle, which expresses a single or predominant RyR (O'Brien et al., 1993), have revealed that the RyRs in this muscle are arranged so that they differ in their potential to physically interact with another protein, the dihydropyridine receptor (DHPR) (Block et al., 1988; Franzini-Armstrong and Jorgensen, 1994). This observation suggests the existence of two types of RyRs, distinguished by the protein species with which they associate, that may differ in the manner in which they are activated to release Ca^{2+}.

Biochemical evidence for the co-expression of two molecularly distinct RyR isoforms, which were termed α and β, was obtained for nonmammalian vertebrate skeletal muscles from chickens, frogs, and fish (Airey et al., 1990, 1993c; Olivares et al., 1991; Lai et al., 1992; Murayama and Ogawa, 1992; O'Brien et al., 1993, 1995). Subsequently, data from RNase protection and in situ hybridization analyses (Giannini et al., 1992, 1995; Takeshima et al., 1995), reverse transcriptase-polymerase chain reaction studies (Ledbetter et al., 1994), Western blot analysis (Conti et al., 1996), and functional studies of muscles from dyspedic mice in which expression of the primary skeletal muscle RyR isoform (RyR1) has been eliminated by homologous recombination (Takeshima et al., 1994a, 1995), indicate that two RyR isoforms, RyR1 and RyR3, are co-expressed in several mammalian muscle and nonmuscle tissues. A striking difference exists in the relative quantities of the two RyRs co-expressed in different vertebrate skeletal mus-

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cles. Similar and abundant levels of the αRyR and βRyR isoforms are found in many, but not all, nonmammalian vertebrate muscles, whereas the βRyR or RyR3 isoforms are expressed at much lower levels than the αRyR or RyR1 isoforms in some nonmammalian vertebrate muscles and in the mammalian muscles studied to date (Conti et al., 1996). The expression of these proteins at markedly different levels may be a rule for mammalian tissues, because it has proven difficult to detect RyR3 protein in them using either immunostaining or Western blot analysis.

Several observations indicate that both of the RyR isoforms co-expressed in nonmammalian vertebrate and mammalian muscles can function as Ca^{2+} release channels. Studies of purified avian αRyRs and βRyRs conducted in vitro demonstrate that both isoforms contain Ca^{2+} channels, but that they differ in their gating properties (Percival et al., 1994). Investigations of the ryanodine-sensitive channel activities in frog and fish SR membranes yield similar conclusions concerning the αRyRs and βRyRs expressed in these species (Bull and Marengo, 1993; O'Brien et al., 1995). Avian and frog αRyRs and βRyRs also differ in the extent to which they are phosphorylated by the cyclic adenosine monophosphate-dependent protein kinase and the Ca^{2+}/calmodulin-dependent protein kinase II, and the avian isoforms differ in the extent to which they bind calmodulin, indicating that the channel activities associated with these proteins have the potential to be regulated differently (Airey et al., 1993c). Moreover, the avian αRyRs and βRyRs are expressed initially at different times during embryonic muscle development (Sutko et al., 1991), which also suggests that the isoforms have unique functions. Like the avian RyR isoforms, both the RyR1 and RyR3 isoforms expressed in mouse skeletal muscle can serve as SR Ca^{2+} release channels (Takeshima et al., 1994a, 1995). This is demonstrated by data from two genetically altered animals, Crooked Neck Dwarf (cn) mutant chickens and dyspedic mice, which indicate that the avian βRyR and the mammalian RyR3 isoform release SR Ca^{2+} in situ (Airey et al., 1993a,b; Ivanenko et al., 1995; Takeshima et al., 1995). Cn/cn and dyspedic muscles are null for the αRyR and RyR1 isoform, respectively, yet ryanodine-sensitive Ca^{2+} transients and contractions can be elicited from both by electrical stimuli or caffeine. This Ca^{2+} release can be attributed to the βRyR and RyR3 isoforms expressed in the mutant muscles. The absence of the αRyR or the RyR1 isoforms in these mutant animals results in a skeletal muscle dysgenesis that is lethal in both cases (Airey et al., 1993a,b; Takeshima et al., 1994a).

RyRs can be localized to specific cellular sites in different cell types, and, in some cases, more than one RyR isoform may be co-expressed within the same cell. Thus, important questions exist as to the intracellular distributions of these proteins and the functional contributions made by each isoform in different tissues. The distributions of RyRs within a cell are determined most directly by immunostaining with RyR isoform-specific antibodies. However, a limitation of this method is that it does not permit RyRs that are active as Ca^{2+} release channels to be distinguished from those that are not functionally active, e.g., ones being synthesized, trafficked, or degraded. For example, RyR and inositol trisphosphate (IP_3) receptors colocalize to RER membranes in the soma of avian cerebellar Purkinje neurons, but exhibit a differential distribution in the endomembranes in the dendrites and dendritic spines in these neurons (Walton et al., 1991; Sharp et al., 1993). These observations lead to the question of whether the RyRs in either or both of these locations are active as Ca^{2+} release channels. Similarly, functionality is an issue when the potential contributions made by different RyR populations is investigated in cell types that co-express more than one RyR isoform.

An approach to this question is to use a probe that only recognizes active RyR channels. Ryanodine binds with high affinity to the RyRs when they are in the conformation associated with an open state of the channel and, therefore, has the potential to serve as such a probe. Moreover, ryanodine can be labeled at the 21-position with bulky substituents, such as the fluorophore Bodipy (**125**), without a significant decrease in high affinity binding (Welch et al., 1994). Thus, at least in theory, comparison of the distribution of RyRs labeled by anti-RyR antibodies with that obtained for RyRs labeled by a fluorescent ryanodine derivative under appropriate cellular activation states has the potential for identifying RyRs that are active as Ca^{2+} release channels. Moreover, the use of a fluorescent group such as eosin, which is an efficient producer of free radicals, would permit utilization of the photo-oxidation labeling approach developed by Deerinck et al. (1994) and detection of active RyRs at the EM level. In practice, the use of fluorescently labeled ryanoids will require the development of conditions that minimize nonspecific binding of these derivatives. If the latter can be achieved, it will be of interest to develop fluorescent ryanodine derivatives that bind in a RyR isoform-specific manner (see next paragraph).

In many cell types, the role of RyR-mediated Ca^{2+} release events is not well understood (Sutko and Airey, 1996). Moreover, in cases in which two RyRs are co-expressed or a single RyR isoform is localized to different regions of the cell, it is unclear whether (and how) the different RyR populations interact to generate a Ca^{2+} signal. A pharmacological approach to these questions will not only be useful, but offers a significant advantage for dissecting the roles of co-expressed RyRs. As discussed below, the large size of the RyRs suggests they may have functions in addition to serving as Ca^{2+} release channels. The use of genetic techniques, such as homologous recombination or anti-sense, to prevent expression of a RyR, eliminates the physical presence, as

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well as the function, of the protein. Therefore, this approach may result in changes that are not simply due to the absence of RyR-mediated Ca^{2+} release events. For example, properties other than the activity of the RyRs as Ca^{2+} channels may influence aspects of cellular organization, such as the localization of other proteins. A more straightforward approach is to use RyR isoform-specific modifiers to alter the channel function of a RyR in a predictable manner under otherwise normal in situ conditions.

Definition of the contributions made by populations of the same RyR isoform that are localized to different sites within a cell, may prove in a practical sense to be a more difficult problem. Resolution of this issue is likely to require the use of several approaches, such as selectively disrupting the mechanism(s) used to activate the RyR channels in each population, the use of Ca^{2+} indicators localized to different regions in the cell (Etter et al., 1996), of spatially restricted techniques for sampling changes in Ca^{2+} (Cheng et al., 1993; Escobar et al., 1994), and of kinetic analyses to dissect the contributions made by each RyR population to spatially averaged changes in cell Ca^{2+}.

C. Ryanodine receptor-molecular properties

With the possible exception of the truncated version of RyR1 expressed in mammalian brain described above, the vertebrate RyRs identified to date are homotetramers comprised of subunit polypeptides with molecular masses of 500 to 600 kDa (McPherson and Campbell, 1993; Meissner, 1994; Ogawa, 1994; Coronado et al., 1994). The massive size of the RyRs may make them physically the largest ion channels. Their closest relatives, the IP_3 receptors, are a little more than one-half as large (Mignery et al., 1989; Nakagawa et al., 1991; Südhof et al., 1991). The RyR isoforms are similar in their overall topology and in that the ion channel forming membrane spanning regions are highly conserved and appear to be localized to the carboxyl terminal 20% of the protein. This is consistent with the size of most other ion channel proteins. In each case, the remaining amino-terminal region of the protein, which consists of more than 80% of the mass of the RyRs, forms a large cytoplasmic foot domain that assumes a quarterfoil shape (Inui et al., 1987; Saito et al., 1988; Lai et al., 1988; Radermacher et al., 1992, 1994; Serysheva et al., 1995).

The three-dimensional topology of the RyR1 isoform has been established in greater detail using cryo-electron microscopy (Radermacher et al., 1994; Serysheva et al., 1995, 1996; Wagenknecht and Radermacher, 1995). These latter studies have yielded reconstructed images that exhibit a four-fold symmetry consistent with the homotetrameric organization of the native protein. An open region extends through the central region of the molecule and may represent the ion conducting channel. In some reconstructions, this pore appears to be plugged at the SR lumenal surface of the RyR, perhaps representing a closed state of the channel. Consistent with this possibility, this plug was not observed in RyRs frozen under conditions that result in channel activation (Radermacher et al., 1994; Serysheva et al., 1995, 1996; Wagenknecht and Radermacher, 1995; Orlova et al., 1996). The upper part of the cytoplasmic domain of the RyR is loosely packed and contains a large percentage of solvent space. As it enters this region of the receptor, the central pore becomes less well defined. The lattice-like structure suggests that each of the subunits may have the potential to undergo significant changes in conformation. Such changes could be involved in the transmission of activation signals received at the cytoplasmic surface of the RyR to the domains of the protein involved in channel gating, which may be located in regions of the molecule close to the SR lumen. Consistent with this scenario, binding sites for agents that affect RyR channel activity, such as calmodulin and the 12 kDa FKBP binding protein (FKBP-12), have been localized to the surface regions of the cytoplasmic domain (Wagenknecht et al., 1994, 1996). In addition, as summarized in detail in a recent review (Ogawa, 1994), putative binding domains have been identified for several regulators of the RyR channel within the context of the primary structure of the RyR. These include Ca^{2+}, adenine nucleotides, and calmodulin. The effects of these agents on RyR channel properties have extensive interactions with each other. For example, calmodulin activates the RyR1 channel in the presence of submicromolar Ca^{2+}, but inhibits channel activity when Ca^{2+} is increased into the micromolar range (Ikemoto et al., 1995; Tripathy et al., 1995). The presence of adenine nucleotides increases the extent to which the RyR1 and avian αRyR isoforms can be activated by Ca^{2+} in vitro (Smith et al., 1988; Percival et al., 1994). In contrast, under similar conditions, the RyR2 and avian βRyR isoforms are activated to a greater extent by Ca^{2+} in the absence of adenosine triphosphate (ATP) (Anderson et al., 1989).

As noted in the preceding paragraph, the massive size of the RyRs has also permitted visualization of two proteins that modify RyR channel properties, calmodulin and FKBP-12, bound to the surface of the receptor. It may be possible to extend this analysis to map the binding sites for non-protein ligands of the RyR, such as ryanodine. The latter will depend though on the nature and the position of the binding site within the structure of the protein and whether a suitable electron dense group can be attached to the ligand without inhibiting its ability to bind to the RyR. In any case, when used in conjunction with information from primary RyR sequence analysis and site-directed mutagenesis, this mapping approach should establish landmarks for determining how the primary sequence of the RyR is folded to achieve the secondary and tertiary structures of the native protein. In addition, this approach should yield important insights into how different domains of the RyR interact to regulate channel activity.

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The importance of the large size of the RyRs is not well understood. Although they are found in a variety of tissue and cell types (Sutko and Airey, 1996), most of our knowledge of RyR structure and function comes from studies of skeletal and cardiac muscles. The RyRs in these muscles are localized primarily to membrane junctions and, in particular, to the space formed by the apposition of the SR membrane with either the surface membrane of the cell (peripheral coupling sites) or with the invaginating t-tubular membrane (triad or diad junctions). Within the context of the junctional structure, the large size of the RyRs may be needed to permit these proteins to participate in the organization of the junction, and/or to span the junctional gap and interact with components of the surface or t-tubular membranes. For example, recent studies indicate that the cytoplasmic loop between the SII and SIII transmembrane repeats in the α_1-DHPR subunit is one such component (Tanabe et al., 1990; Lu et al., 1994, 1995; El-Hayek et al., 1995a). However, functional RyRs may be localized outside of the junctional structures in both cardiac and skeletal muscles (Jorgensen et al., 1993; Carl et al., 1995). Moreover, it is unclear whether the RyRs expressed in nonmuscle tissues exist within a structural format that necessitates their large size. Thus, it will be of interest to learn whether the significant mass of this protein has evolved to give the RyR channel specific functional and/or regulatory properties. Expression of RyRs having N-terminal truncations in homologous systems, such as those produced by genetic knockouts (Takeshima et al., 1994a; Nakai et al., 1996), should provide insights into the functional significance of the large size of these proteins.

D. Effects of ryanodine

Ryanodine and related compounds have complex effects on the conductance and the gating of single RyR channels. Studies with skeletal muscle, particularly those conducted by Fairhurst and Hasselbach (1970) and by Fairhurst (1974), and the work of Hilgemann (Hilgemann, 1983, 1986) using cardiac muscle preparations demonstrated that the actions of ryanodine result in an increase in the Ca^{2+} permeability of the SR. In addition, ryanodine can decrease SR Ca^{2+} permeability (Sutko et al., 1979, 1985; Jones et al., 1979; Fabiato, 1985; Fleischer et al., 1985; Meissner, 1986; Lattanzio et al., 1987). Therefore, it is not surprising that, once the RyR was identified, ryanodine and related compounds were found to have complex effects on the conductance and gating of its channel.

Three general effects on the activity of RyR channels have been observed for ryanodine. At submicromolar concentrations, it has been reported to increase channel activity with openings to a full conductance state (Pessah and Zimanyi, 1991). It should be noted that this change has not been observed as consistently as the two effects described next. Also, at submicromolar concentrations, ryanodine causes the channel to exhibit partially conducting or subconductance states (Rousseau et al., 1987). This effect is observed consistently and has become a signature for a ryanodine-modified RyR channel. Multiple subconductance levels have been observed (Buck et al., 1992; Liu et al., 1989; Kwok and Best, 1990; Pessah and Zimanyi, 1991; Ding and Kasai, 1996), but one that is ~ 50% of the full conductance level is the most common. Both of the first two effects would contribute to the ryanodine-induced increase in SR Ca^{2+} permeability noted above. At micromolar or greater concentrations, ryanodine produces a closed state of the channel (Meissner, 1994). This effect is also consistently observed and accounts for the decrease in SR Ca^{2+} permeability caused by ryanodine. When defined in single channel experiments, the concentration dependence of these effects by ryanodine must be considered as estimates, because an insufficient number of trials have been conducted to accurately determine the concentration of ryanodine binding when a single RyR protein is involved.

It is not clear whether ryanodine alters the conductance state of the RyR channel by stabilizing a specific conformation of the channel via allosteric effects, or whether it physically interferes with the flux of ions through the pore of the channel. As discussed below in Section V., recent data obtained from analysis of the effects of ryanoids on the conductance of the RyR2 channel favor an allosteric mechanism (Welch et al., 1997); however, additional studies are needed to resolve this issue.

Given the complexities of the effects by ryanodine on RyR channel function, it is not surprising that investigations of the binding of this ligand to RyRs have yielded complex results. There is general agreement that [^{3}H]ryanodine binds to sites on the RyRs exhibiting high (K_D ~1 to 10 nM) and low (K_D ~1 to 10 μm) affinities. It is also generally accepted that high affinity binding results in RyR channel activation and in channels exhibiting conductance substates, and that low affinity binding causes channel inhibition. The functional consequences of ryanodine binding exhibit use-dependence. For example, following infusion of ryanodine into rats, contracture developed in muscles receiving electrical stimuli, but not in their nonstimulated counterparts (Procita, 1956). Moreover, the latter muscles showed no signs of having been exposed to ryanodine if this agent was washed out before activation of the muscle. This indicated that ryanodine did not bind to RyRs in the inactive muscle. Consistent with this finding, high affinity [^{3}H]ryanodine binding is observed under conditions that are associated with activation of the RyR channel, indicating that ryanodine binds to a RyR conformation associated with an open state of the channel. This property has proven useful, because it permits [^{3}H]ryanodine binding to be used as an index of channel activation (Hawkes et al., 1992; Meissner and El-Hashem, 1992).

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Several observations indicate that the tetrameric form of the RyR is required to bind ryanodine with high affinity. This is demonstrated by the inability of the individual subunits dissociated by exposure of the tetramer to the detergent Zwittergent 3–14, instead of 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (which maintains the RyR as a tetramer) to bind [^{3}H]ryanodine (Lai et al., 1989). A caveat concerning the latter studies is that it was not shown that the separated monomers could be reassembled to form a tetrameric RyR capable of binding ligand. Consequently, denaturation of the monomer may have masked its ability to bind ligand. Also, it has been found that under appropriate conditions, the use of covalent cross-linking to stabilize the RyR tetramer does not interfere with high affinity ryanodine binding, indicating further that the tetrameric form of the RyR is relevant for ligand binding (Lai et al., 1989; Carroll et al., 1991; Shoshan-Barmatz et al., 1995). The inability of RyR monomers to bind ryanodine suggests that the binding site is created by the correct juxtaposition domains contributed by more than one subunit, or alternatively, that each monomer may contain a binding site, but the site only assumes the conformation appropriate for ligand binding in the context of the molecular organization achieved in the tetramer.

Results obtained for [^{3}H]ryanodine binding to purified RyRs suggest stoichiometries (moles of [^{3}H]ryanodine/mole of RyR tetramer) of 1:1 for the high affinity binding process (Lai et al., 1988, 1989; Carroll et al., 1991; Pessah and Zimanyi, 1991; Wang et al., 1993). Stoichiometries of either 3:1 (Lai et al., 1989) or 1:1 (Wang et al., 1993) have been reported for low affinity ryanodine binding. This difference in the number of low affinity sites has led to two different models of ryanodine binding to its receptor. The first model involves four initially identical interacting binding sites per RyR tetramer that can serve as either high or low affinity sites (Lai et al., 1989; Carroll et al., 1991; Pessah and Zimanyi, 1991) and will be termed the interconvertible site model (ISM). The second model proposes two nonidentical or distinct interacting ryanodine binding sites per RyR tetramer that subserve high and low affinity ryanodine binding, respectively (Wang et al., 1993). The model will be termed the distinct site model (DSM). In the ISM, the four sites are considered initially to be equivalent and capable of binding ryanodine with high affinity when the RyR assumes a conformation associated with an open state of the channel. The binding of ryanodine to one site exerts a negatively cooperative effect on the remaining sites, lowering their affinity for ryanodine. In one version of the ISM, binding to the first site produces an equivalent reduction in the affinity of the remaining three sites (Carroll et al., 1991). Binding of ryanodine to all three low affinity sites leads to channel closure. In a second version of the ISM, a sequential process is proposed wherein binding of ryanodine to each site successively lowers the affinity of the remaining unbound sites, leading to four classes of ryanodine binding sites having different K_D values (Pessah and Zimanyi, 1991). Binding of ryanodine to each site is also proposed to alter successively the conductance of the RyR channel to lower and lower fractional values of the fully conducting state. Ultimately, this leads to stabilization of a closed state of the channel, when all four sites have bound ryanodine.

In the DSM, two distinct classes of binding sites that differ in their affinity for ryanodine exist in each RyR tetramer at a stoichiometry of 1:1. As in the ISM, binding of ryanodine to the high affinity site requires the RyR to assume a conformation associated with an open state of the channel and stabilizes the protein in a conformation exhibiting a fractional conductance. Ryanodine binding to the low affinity site results in channel inhibition. Because the two types of ryanodine binding sites have distinct properties, it is not necessary to have negatively cooperative interactions between sites in the DSM to produce low affinity binding. An intersite interaction that is part of this model is the binding of ryanodine to the low affinity site that leads to a decrease in the rate of dissociation of ryanodine bound to the high affinity site (McGrew et al., 1989; Wang et al., 1993). The nature of this effect, e.g., whether it (*a*) involves steric interactions and a physical trapping of ryanodine bound to the high affinity site or (*b*) allosteric interactions between different regions of the RyR, remains to be determined.

A key difference between the DSM and ISM is that, in the DSM, the same physical sites do not subserve both high and low affinity ryanodine binding. In both the DSM and ISM, high and low affinity ryanodine binding is affected by conformational changes in the RyR, and, in both models, these conformational changes are a function of the activation state of the channel. In addition in the ISM, conformational changes producing a negatively cooperative effect are also a function of the extent of ryanodine binding. In comparing the DSM and ISM, it should be considered that determinations of the properties of binding sites with K_D values in the micromolar range using filtration assays can have a degree of uncertainty.

Additional experimentation is required to establish the number and the identity of the ryanodine binding sites. One approach that is being taken to resolve this issue is to identify the binding sites within the context of the primary sequence of the RyR. In this regard, significant advances have been made recently, and two lines of evidence indicate that both ryanodine binding site(s) may be localized to the C-terminal 76 kDa of the receptor. In an elegant series of studies, Callaway et al. (1994) took advantage of the ability of ryanodine binding to a low affinity site(s) to stabilize ryanodine bound to the high affinity site. Binding to the latter sites was maintained following proteolysis with trypsin to yield a 76-

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kDa fragment that was derived from the carboxy terminus of the RyR monomer. Independently, Witcher et al. (1994) used 10-O-(3-[4-azidobenzamido]propanoyl)ryanodine (**84B**), a photolabile cross-linking derivative of ryanodine, to covalently bind tritiated ligand to the RyR under conditions that resulted in high affinity binding. Again, a 76-kDa fragment derived from the C-terminus of the receptor was cross-linked. There can be some uncertainty associated with the approach used in the latter study, as regions not involved in ligand binding may be cross-linked. However, the agreement reached by both of the above complementary approaches indicate strongly that at least a significant part of the high affinity and presumably also the low affinity ryanodine binding sites are localized as indicated by these studies. Further utilization of such approaches to the site(s) responsible for low affinity ryanodine binding will permit a more precise determination of the physical relationship existing between the high and low affinity binding sites. For example, the studies by Callaway et al. (1994) suggest that at least part of the low affinity ryanodine binding site is present in the C-terminal 76 kDa fragment of the RyR, because high concentrations of ryanodine still were able to slow the dissociation of ryanodine bound to the high affinity site in this fragment.

A pharmacological approach to identifying the number and nature of the ryanodine binding sites could be taken if ligands specific for either the high or low affinity site(s) were available. For example, in contrast to ryanodine, which both increases and decreases SR Ca^{2+} permeability at nanomolar and micromolar concentrations, respectively, the ryanoid designated as the ester A (**65**) (Ruest et al., 1985) only causes an increase in the Ca^{2+} permeability of skeletal muscle SR membranes at concentrations up to 3 mM (figs. 1–3, and data not shown) (Sutko et al., 1990). The ester A binds to the high affinity ryanodine binding site with a K_D = 110 nM (Welch et al., 1994). If this ryanoid is shown not to bind to the low affinity site, e.g., by using [^{3}H]ester A, the selective nature of the effects of this agent on SR Ca^{2+} permeability would suggest one of two possibilities. The first possibility is that the high and low affinity binding sites are distinct entities, as proposed in the DSM. In this case, ryanodine should still bind to the low affinity sites in the presence of the ester A. A second possibility is that the binding of the ester A does not lead to the conformational change necessary to induce the negative cooperativity required to convert high affinity to low affinity sites, as described in the ISM. In this case, the binding of ryanodine to both the high and low affinity sites should be attenuated by pretreating the RyRs with the ester A. Ryanodine binding to the high affinity site would be reduced due to direct competition between ryanodine and the ester A. The number of low affinity sites available to bind ryanodine would be decreased by the inability of ester A binding to the high affinity site to produce the conformation change necessary to create these sites.

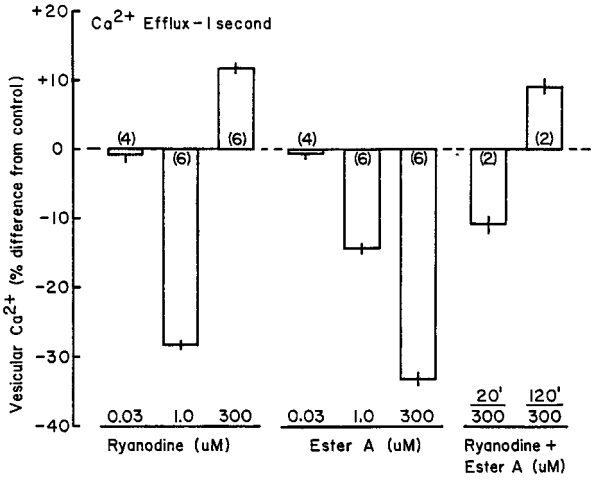

FIG. 1. The effects of ryanodine and the ester A on the passive efflux of Ca^{2+} from skeletal muscle terminal cisternae vesicles. Membrane protein (5 mg/ml) was suspended in a solution containing 0.1 M KCl, 10 mm Pipes/Tris (pH 6.8) and 0.1 mM $^{45}CaCl_2$ and incubated for 2 h at 37°C in the absence or presence of the concentrations of ryanodine and the ester A indicated along the abscissa. Ca^{2+} efflux occurring during 1-sec intervals was measured at 25°C by diluting 5 μl of the vesicle suspension into 500 μl of a solution containing 0.1 M KCl, 10 mM Pipes/Tris (pH 6.8). Ca^{2+} efflux was terminated by the rapid addition of 5 ml of an ice-cold solution containing 0.3 M sucrose, 10 μM ruthenium red, 0.5 mM $HgCl_2$, 0.5 mM $LaCl_3$ and 10 mM Pipes/Tris (pH 6.8). The vesicles were trapped on Whatman GF/A filters and washed with two additional 5-ml aliquots of the terminating solution. The ^{45}Ca retained on the filters was measured using liquid scintillation counting techniques. The quantity of ^{45}Ca present in the vesicles after the efflux period was expressed as a percentage of that present before the initiation of the efflux. The difference between the values obtained for the preparations treated with ryanodine and ester A and for nontreated vesicles are presented in this figure. Control vesicles retained 61.45 ± 1.48% of their initial Ca^{2+} content after the 1-sec efflux period, and the initial vesicular content of Ca^{2+} was not affected by either ryanodine or the ester A. Values more negative and more positive than the control values are indicative of enhanced and inhibited effluxes, respectively. Similar results were obtained when ryanodine or the ester A were present in the efflux medium. The effects of coadministration of ryanodine and the ester A were assessed 20 and 120 min after the simultaneous addition of a 300 μM concentration of each agent. The values shown are means ±standard error for the number of preparations indicated within the bars. This figure is taken from Sutko et al. (1990) with the permission of the Plenum Publishing Corporation.

The potential for using a pharmacological approach, such as that described in the preceding paragraph, to investigate the properties of ryanodine binding sites is supported further by the finding that other ryanoids also differ in their relative abilities to increase and decrease the Ca^{2+} permeability of SR membranes. For example, Besch and coworkers (Humerickhouse et al., 1993, 1994; Gerzon et al., 1993; Bidasee et al., 1995) have found that the ratios of the concentrations required to increase and decrease the Ca^{2+} permeability of SR membranes vary between ryanoids. Moreover, Mitchell et al. (1996) found that the EC_{50} concentration of a ryanoid required to

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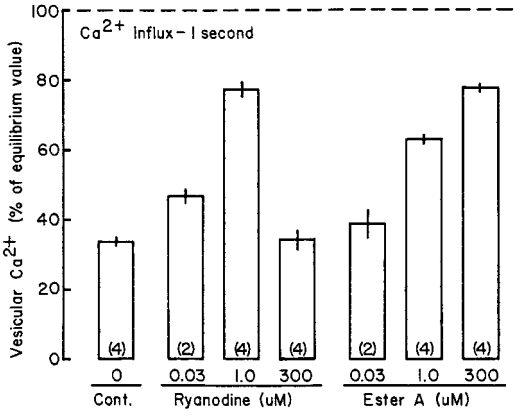

FIG. 2. The effects of ryanodine and the ester A on the passive influx of Ca^{2+} into skeletal muscle terminal cisternae vesicles. Membrane protein (5 mg/ml) was suspended in a solution containing 0.1 M KCl, 10 mm Pipes/Tris (pH 6.8) and was incubated for 2 h at 37°C in the absence and presence of ryanodine and the ester A at the concentrations shown along the abscissa. The quantity of Ca^{2+} entering the vesicles during a 1-sec period was determined at 25°C by mixing 46.3 μl of the protein suspension with 3.6 μl of $^{45}CaCl_2$. The final concentration of Ca^{2+} was either 0.1 or 1.0 mM. The results obtained with either concentration were similar once normalized and have been combined. Ca^{2+} influx was terminated, and vesicular Ca^{2+} was measured as described for figure 1. The quantity of Ca^{2+} entering the vesicles during the 1-sec uptake interval is expressed as a percentage of the equilibrium vesicular Ca^{2+} content obtained after 20 min in the presence of the Ca^{2+} ionophore, ionomycin (1 μM). Neither ryanodine nor the ester A affected the latter quantity. The values presented are means ± standard error for the number of preparations shown in each bar. This figure is taken from Sutko et al. (1990) with the permission of the Plenum Publishing Corporation.

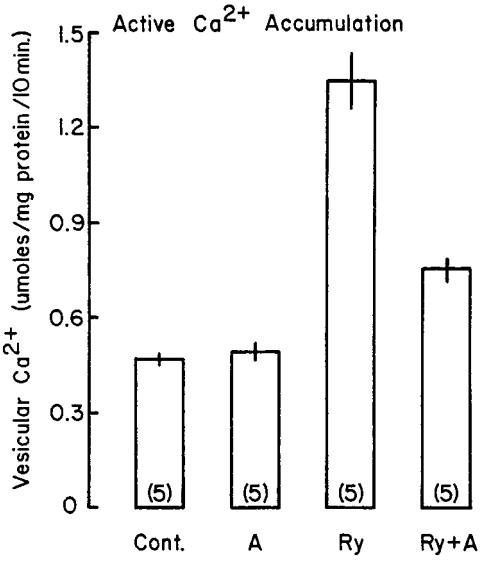

FIG. 3. The effects of ryanodine (Ry) and the ester A (A) on ATP-dependent Ca^{2+} accumulation by skeletal muscle terminal cisternae membranes. Membrane protein (0.2 to 0.8 mg/ml) was added to a medium containing 50 mM histidine (pH 7.0), 3 mm $MgCl_2$, 5 mM ATP, 3 mM K oxalate, 100 mM KCl and 38 μM ^{45} $CaCl_2$; the resulting compound was incubated for 10 min at 37°C in the absence and presence of 300 μM concentrations of ryanodine or the ester A. Ca^{2+} uptake was terminated and vesicular Ca^{2+} measured as described for figure 1. The values shown have been corrected for Ca^{2+} binding occurring in the absence of ATP (which was unaffected by either ryanodine or the ester A) and represent means ± standard error for the number of preparations indicated in the bars. This figure is taken from Sutko et al. (1990) with the permission of the Plenum Publishing Corporation.

cause half-maximal efflux of Ca^{2+} from SR vesicles is similar to the dissociation constants measured for high affinity binding in all cases tested. In contrast, the EC_{50} concentration of ryanoids required to close the channel varied from 5 to 1000 times the high affinity dissociation constants. Recall that when mediated by the RyR, these two effects are thought to involve, respectively, binding to the high and low affinity ryanodine binding sites. Thus, these data suggest that these sites differ in their relative affinities for several ryanoids, a complexity that may be most easily explained by the existence of two distinct classes of binding sites. It has been observed also that exposure of RyR2 channels to one of these compounds, β-alanyl ryanodine (**84A**), precludes inhibition of the channel by a subsequent exposure to high concentrations of ryanodine, a treatment that normally produces this effect (Tinker et al., 1996). This result indicates the complex nature of the interactions between the high and low affinity ryanodine binding sites that will require additional experiments to understand.

Consideration of the functional consequences of ryanodine binding to the RyRs raises the issue of the physiological relevance of the partial conductance states (substates) exhibited by these proteins when their activity is recorded in vitro. It is important to note that in several laboratories, substates are observed in the absence of ryanodine and, therefore, may represent normal conductance modes of the channel that are stabilized by ryanodine binding. In addition, substates are not routinely observed in every laboratory; consequently, the possibility exists that they may be induced by the conditions associated with either sample preparation and/or the experiment. An interesting observation in this regard is that the association of FKBP12 with RyR1 results in a predominance of channel openings to a full conductance level, whereas substates frequently are observed in the absence of the immunophilin (Brillantes et al., 1994). The extent to which different laboratories have studied RyRs without and with bound FKBP12 must be considered.

An important question concerns whether the substate conductances serve as a physiologically important mechanism for regulating the average quantity of Ca^{2+} released per unit time by a RyR. If this is the case, it will be important to define how switching between these conductance modes is regulated in vivo. The latter will require investigations of RyR conductances in situ, and in vitro under conditions known to mimic those existing in situ, where the regulatory state of the protein has been defined.

In summary, several questions exist concerning (*a*) the molecular and biophysical properties of the RyR channels and (*b*) the roles of these proteins in signal

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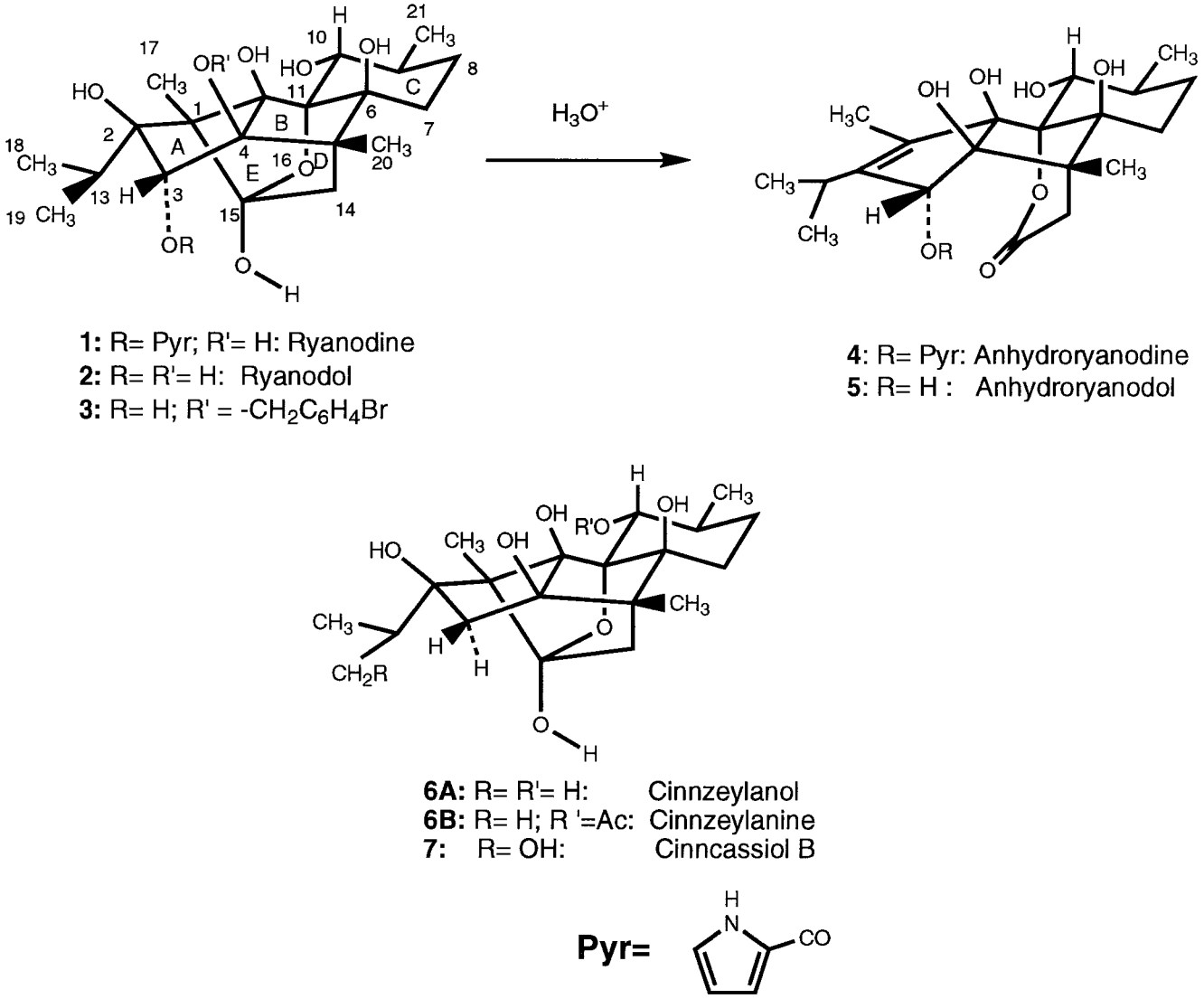

FIG. 4. Dehydration of ryanodine and ryanodol. Diterpenoids from *Cinnamomi cortex*.

transduction systems in different cell types. These questions involve the intracellular distribution of functional RyR channels, the number and nature of ryanodine binding sites, and the manner in which the different RyR isoforms, particularly those that are co-expressed within the same cell, function to generate cellular Ca^{2+} signals. Resolution of these questions will benefit from the development of new pharmacological agents that have binding site-specific and RyR-isoform-specific channel actions and that either activate or inhibit RyR channel activity. In the next section, we consider the chemistry of ryanodine and related compounds and the progress that has been made in achieving selective modifications of the structure of these compounds.

IV. Ryanoids: Ryanodine and Related Compounds

A. Chemistry

Ryanodine (**1**) (fig. 4) is the complex polycyclic, polyhydroxylic diterpene (+)-ryanodol (**2**) esterified at C3 with pyrrole-2-carboxylic acid (see Jenden and Fairhurst, 1969; Jefferies and Casida, 1994, for review). The structure of ryanodine (**1**) was established by Wiesner and colleagues (Wiesner, 1972) more than 25 years ago in what remains a classic example of chemical structure elucidation using chemical degradation. The x-ray analysis of a *p*-bromobenzyl ether derivative (**3**) of ryanodol confirmed this structure with a minor change that the isopropyl and the hydroxyl groups at C2 had to be reversed (Srivastava and Przybylska, 1968) and are arranged as shown in structure **2**.

Ryanodol ($C_{20}H_{32}O_8$) is a pentacyclic diterpene that has a ring skeleton consisting of 14 carbon atoms and a single oxygen atom. For uniformity, the numbering system of ryanodol will be used throughout this review. Four of the rings in ryanodol (A, B, C, and E) are carbo-

[1] The Chemical Abstract name for ryanodol is [3S-(3α,4β,4aS, 6α,-6aα,7α,8β,8aα, 8bβ,9β,9aβ)]-hexahydro-3,6a,9-trimethyl-7-(1-methylethyl)-69-methanobenzo[1,2]pentaleno[1,6-bc]furan-4,6,7,8,8a,8b,9a-(6aH,9H)-heptol.

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cyclic, whereas the fifth ring (D) contains an oxygen atom. There is a methyl group, at C1, and an isopropyl group, at C2, attached to cyclopentane ring A, one tertiary methyl group at C5 in cyclopentane ring B, and a secondary equatorial methyl group at C9 in cyclohexane ring C. There are five tertiary hydroxyl groups at C2, C4, C6, C12, and C15. The hydroxyl at C15 is part of a hemiketal group formed by the tertiary hydroxyl group at C11 and the carbonyl group at C15. There are two secondary hydroxyl groups, one in ring A (α-oriented) at C3 and one in ring C at C10 (α-oriented and equatorial). Many diterpenoids from *Cinnamomi cortex*, known as cinnzeylanols (see **6**) and cinncassiols (see **7**), have a carbon framework closely related to that of ryanodol (**2**) (Nohara et al., 1981).

In acidic medium, ryanodine (**1**) dehydrates easily (Wiesner, 1972) (fig. 4) and gives anhydroryanodine (**4**), losing at the same time its typical biological activities (Pessah et al., 1985; Waterhouse et al., 1987; Jefferies et al., 1991, 1992b). The loss of water is accompanied by an important modification in the skeleton of the molecule. Anhydroryanodine (**4**) and anhydroryanodol (**5**) were important degradation products in the elucidation of the structure of ryanodine (Wiesner, 1972). As described below in this section, anhydroryanodol was a relay compound in the total synthesis of ryanodol (**2**) (as described in the next section and illustrated in fig. 8).

The ryanodine molecule can be viewed as having a hydrophilic face bearing five hydroxyl groups at C2, C4, C6, C12, and C10 and a lipophilic surface formed by the isopropyl group and the hydrogens attached to C14, C20, C7, C8, and C21. Both faces can be extended or shortened by a proper orientation of the pyrrole ester group at C3 (Jefferies et al., 1991, 1992b).

B. Progress Toward the Synthesis of Ryanodine

The development of a synthetic scheme for a biologically active natural product, such as ryanodine, offers the following advantages: (*a*) it is a potential source for the compound, because the natural source has become scarce; (*b*) it provides insights into how the compound may be made in vivo; and (*c*) it yields intermediates with partial structures that can be used to establish the minimal structure required for the biological activity of the parent compound and to make chemical derivatives with different functional properties. In addition, synthesis of molecule with the complexity of ryanodine offered a significant challenge in the field of synthetic organic chemistry.

The total synthesis of (+)-ryanodol (**2**) was accomplished by Deslongchamps, Ruest, and colleagues in the late 1970s (Belanger et al., 1979; Deslongchamps et al., 1990) and remains an elegant and unique achievement in molecular construction. The strategies and methodologies involved in this synthesis have been described recently in detail (Deslongchamps et al., 1990). The starting materials for this synthesis are the activated diene **12** obtained in 11 steps from vanillin (**10**) (fig. 5) and the optically active dienophile **9** obtained in eight steps from S-(+)-carvone (**8**). It should be noted that vanillin has been isolated from extracts of *Ryania speciosa Vahl* (Ruest and Deslongchamps, unpublished observations). The synthetic fate of each of the carbon atoms in structures **9** and **12** has been shown by numbering them using the numbering system of ryanodol.

The Diels-Alder reaction of spirolactone dienone **12** and enone **9** provided a mixture of four diastereoisomeric adducts—**13A** and **B**, and **14A** and **B**—in quantitative yield. Sequential treatments of the mixture in basic media (to give **15** endo-exo and **16** endo-exo, fig. 6), acidic media, and then basic media, led to the optically active pentacyclic aldehyde **17** that was stored as its protected form **19**. Diastereoselective formation of the desired isomer **17** from the precedent mixture has been explained by steric hindrance between the isopropyl group and the 1,2-diol moiety that prevented the formation of the undesired diastereoisomer **18** during the last step, which involves the aldol condensation between C3 and the C4-carbonyl group.

Baeyer-Villiger-retroepoxidation sequence on the pentacyclic olefin-ketone **19** (fig. 7) gave the olefin lactone **20**. Ozonolysis of **20** in protic medium resulted in the formation of the desired aldol condensation product **21** that was transformed into the equatorial monomethylated compound **22**. Subsequent reduction of ketone **22** led selectively to the equatorial alcohol **23**. Protection of the hydroxycarbonate moiety of this compound as a methoxymethyl orthocarbonate group and reduction of the lactone function gave the triol **24** that was selectively oxidized to the hemiketal **25**. The secondary hydroxyl in this intermediate was transformed to mesylate **26**, which underwent a basic medium fragmentation to yield the nine-membered lactone **27** that was immediately hydrolyzed to carbonate **28**. Selective epoxidation of the olefin **28** gave the epoxide **29**, which furnished a six-membered lactone **30** after mild aqueous basic treatment. The triol lactone **30** was converted into the primary *p*-nitrobenzoate derivative **31**, which was oxidized to the ketone **32**. Sequential reduction of this compound and acetylation of the resulting alcohol **33** furnished the equatorial acetate derivative **34**.

Modifications of ring A of compound **34** led to the substituted cyclopentenol moiety of anhydroryanodol (**5**); compound **34** (fig. 8) was first converted to a mixture of enol ethers (**35**) that was oxidized by ozone to cyclopentanone **36**. Transformation of ketone **36** to its enol acetate **37** was followed by a basic treatment that yielded the desired enone **38**. Selective reduction of the C3 carbonyl group gave the endoallylic alcohol **39**. Removal of the carbonate and acetate groups under mild basic aqueous conditions gave a mixture (≈3:1) of anhydroryanodol (**5**, C15-O-C11 bridging) and its regioisomer (**40**, C15-O-C3 bridging) previously named epianhydroryanodol (Belanger et al., 1979; Deslongchamps et al.,

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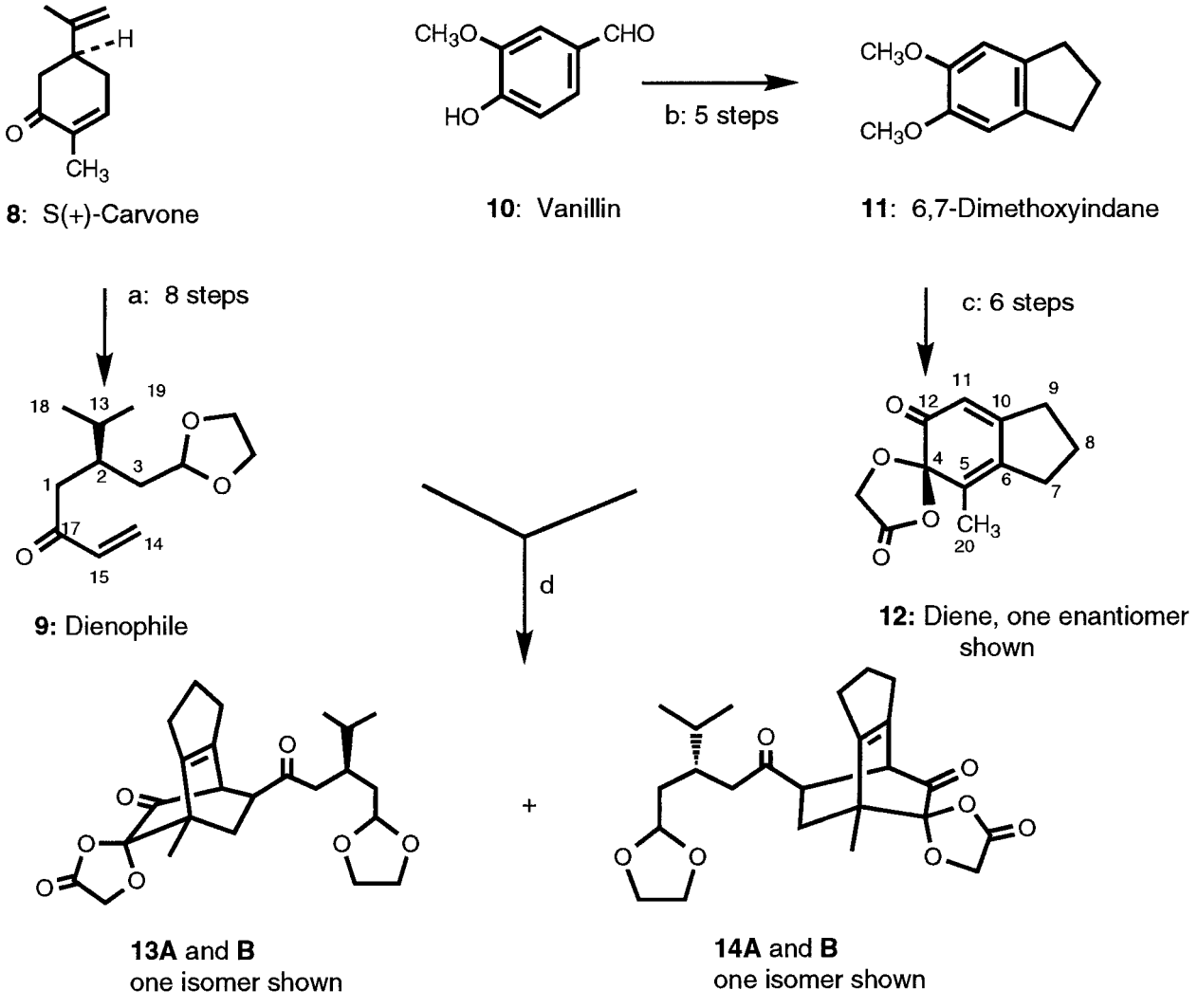

Reaction conditions: a: 1) H_2, Pt, ether; 2) O_3, EtOAc; 3) $(CH_2OH)_2$, p-TsOH; 4) CH_3I, K_2CO_3, acetone; 5) $LiAlH_4$, ether; 6) PCC, CH_2Cl_2; 7) CH_2=$CHMgBr$, ether; 8) CrO_3, H_2SO_4, acetone; b: 1) $(CH_3)_2SO_4$, NaOH; 2) $CH_2(COOH)_2$, C_5H_5N; 3) H_2, Ra/ Ni, EtOH; 4) PPA; 5) Zn/Hg, HCl; c: 1) $SnCl_4$, $BrCH(OCH_3)_2$; 2) BBr_3, CH_2Cl_2; 3) $BrCH_2COBr$, C_5H_5N; 4) K_2CO_3,THF; 5) NH_2NH_2, KOH, $(CH_2OH)_2$; 6) NaOH, NBS; d: reflux, C_6H_6;

FIG. 5. Total synthesis of ryanodol: Diels-Alder adducts from a dienophile and a diene obtained from carvone and vanillin, respectively.

1990). Selective epoxidation of either isomer, **5** or **40**, or of a mixture of both compounds, yielded the anticipated β-epoxide in the form of its C3-lactone **41**, the most stable isomer in that medium. Basic aqueous treatment of **41** led to epoxide **42** of anhydroryanodol, that on treatment with lithium in ammonia gave (+)-ryanodol (**2**). This last sequence implies an attack by a dianion, or a radical anion (see **43,** fig. 9) previously formed at C15 by the reduction of the lactone carbonyl onto the carbon atom at position 1, which opened the oxiran moiety to give the C2-hydroxyl group with the desired stereochemistry.

Attempts to obtain native ryanodine (**1**) from ryanodol (**2**), attempts that require esterification of the latter compound at position 3 with 2-pyrrole carboxylic acid, have been unsuccessful. Models show that selective access to the hindered α-hydroxyl group at C3 is very difficult and may require severe esterification conditions that would not be tolerated by the fragile ryanodol structure (Belanger et al., 1979; Deslongchamps et al., 1990).

It has been shown recently (Ruest and Deslongchamps, 1993) that esterification (Neises and Steglich, 1978) of (+)-ryanodol by pyrrole-2-carboxylic acid yields 10-O-pyrrolecarbonylryanodol (**44**), a compound that retains signif-

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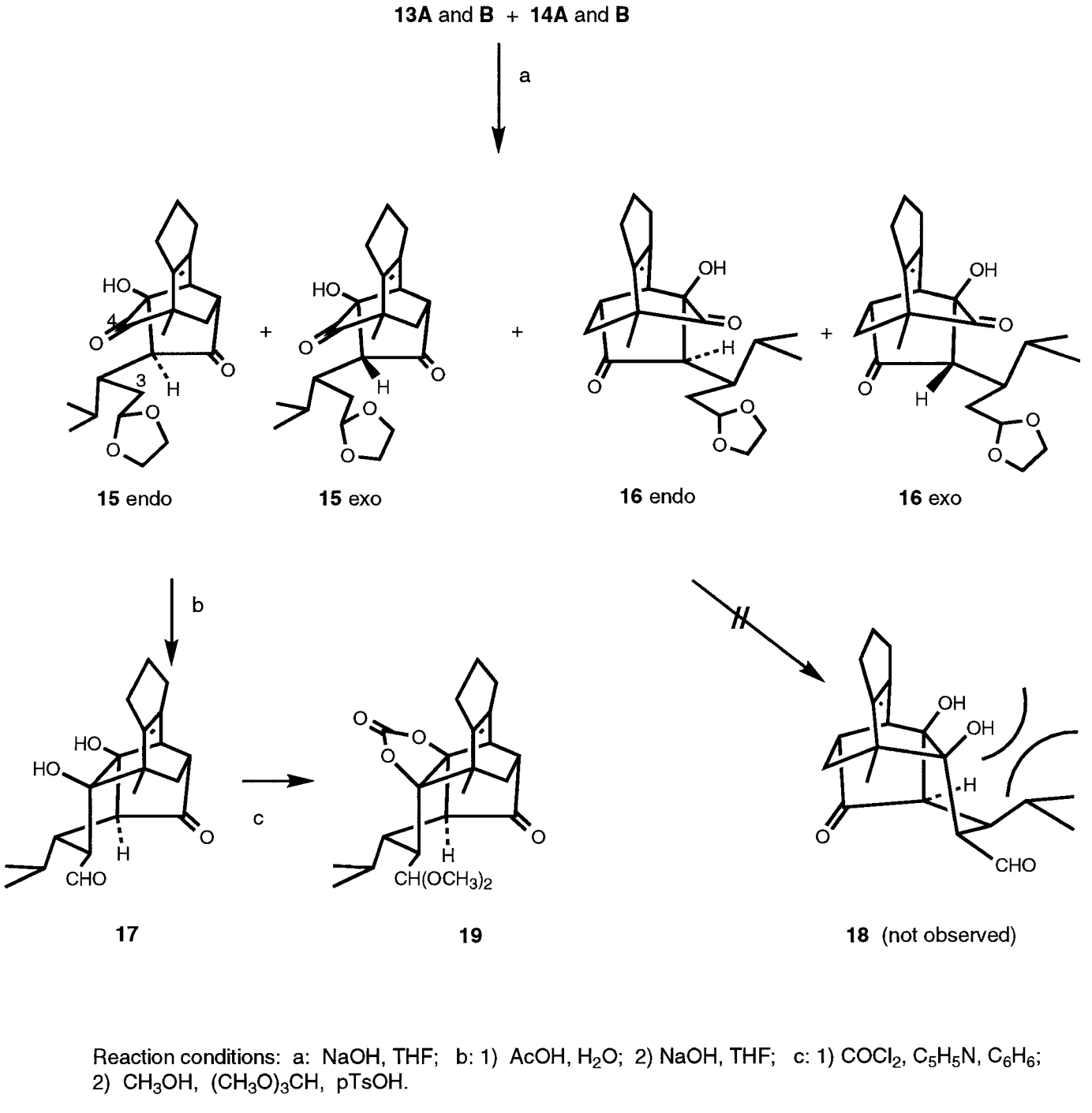

FIG. 6. Total synthesis of ryanodol: formation of the pentacyclic intermediate **17**.

icant binding to the ryanodine receptor and is proving useful as the starting point for a series of compounds that will be used to test the regions of the ryanodine binding site that interact with the pyrrole-2-carboxylic acid moiety (Welch et al., 1996a) (see fig. 17, compounds **2A** through **2J**, and section IV.I.).

C. 3-Epiryanodine Synthesis

The total synthesis of 3-epiryanodine (**50**) (fig. 10) starting from either anhydroryanodine (**4**) or anhydroryanodol (**5**) has been achieved recently (Ruest and Deslongchamps, 1993). Treatment of the starting material with lithium in ammonia gave 2,3-dideoxy-Δ^2-ryanodol (**45**), which yielded 1,2-epoxy-3-epianhydroryanodol (C15-O-C11 bridging) (**48**) upon oxidation with an excess of peracid. The unisolated 2,3-epoxyryanodol (**46**) was unstable in this medium and suffered the well known cleavage to the anhydro series, forming unisolated 3-epianhydroryanodol (**47**) that was readily epoxidized. Treatment of this epoxide with lithium in ammonia re-

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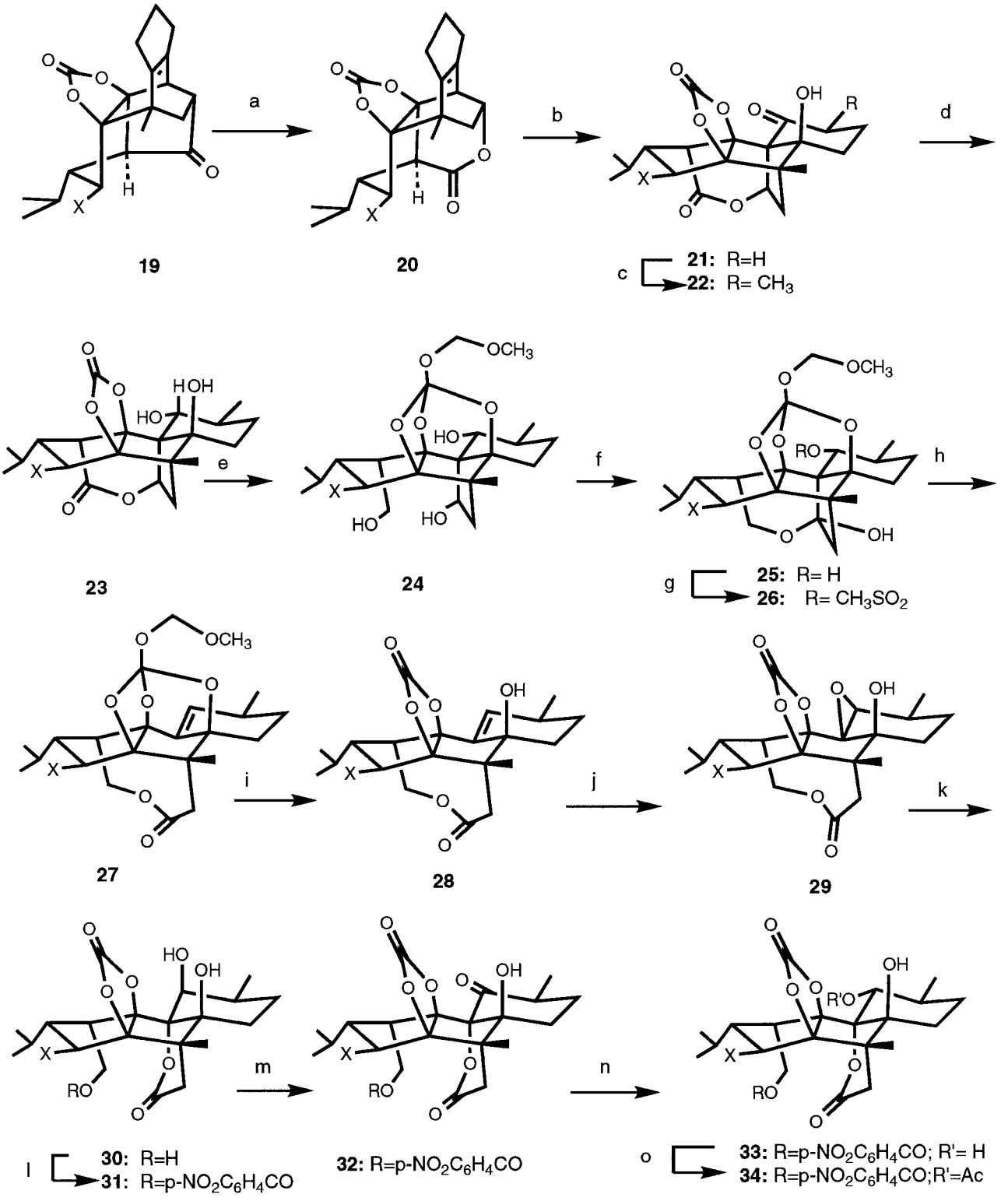

Reaction conditions: a: 1) AcO_2H, AcOH,NaOAc; 2) WCl_6,n-BuLi,THF; b: O_3, p-TsOH,EtOAc; c: LDA, Et_3B,CH_3I; d: $NaBH_4$,THF, MeOH; e: 1)MOMCl, NaH,THF; 2) LAH,THF; f: PCC,CH_2Cl_2, -20°; g: MsCl, C_5H_5N; h:CH_3SOCH_2Li, DMSO; i: HBF_4, H_2O, THF; j: CF_3CO_3H, $NaHCO_3$, $(CH_2Cl)_2$; k: NaOH, H_2O,THF; l: p-$NO_2C_6H_4COCl$, C_5H_5N; m: PCC, CH_2Cl_2; n: $LiBH_4$, THF, -20°; o: Ac_2O, C_5H_5N.

(X= $CH(OCH_3)_2$)

FIG. 7. Total synthesis of ryanodol: preparation of rings B, C and D.

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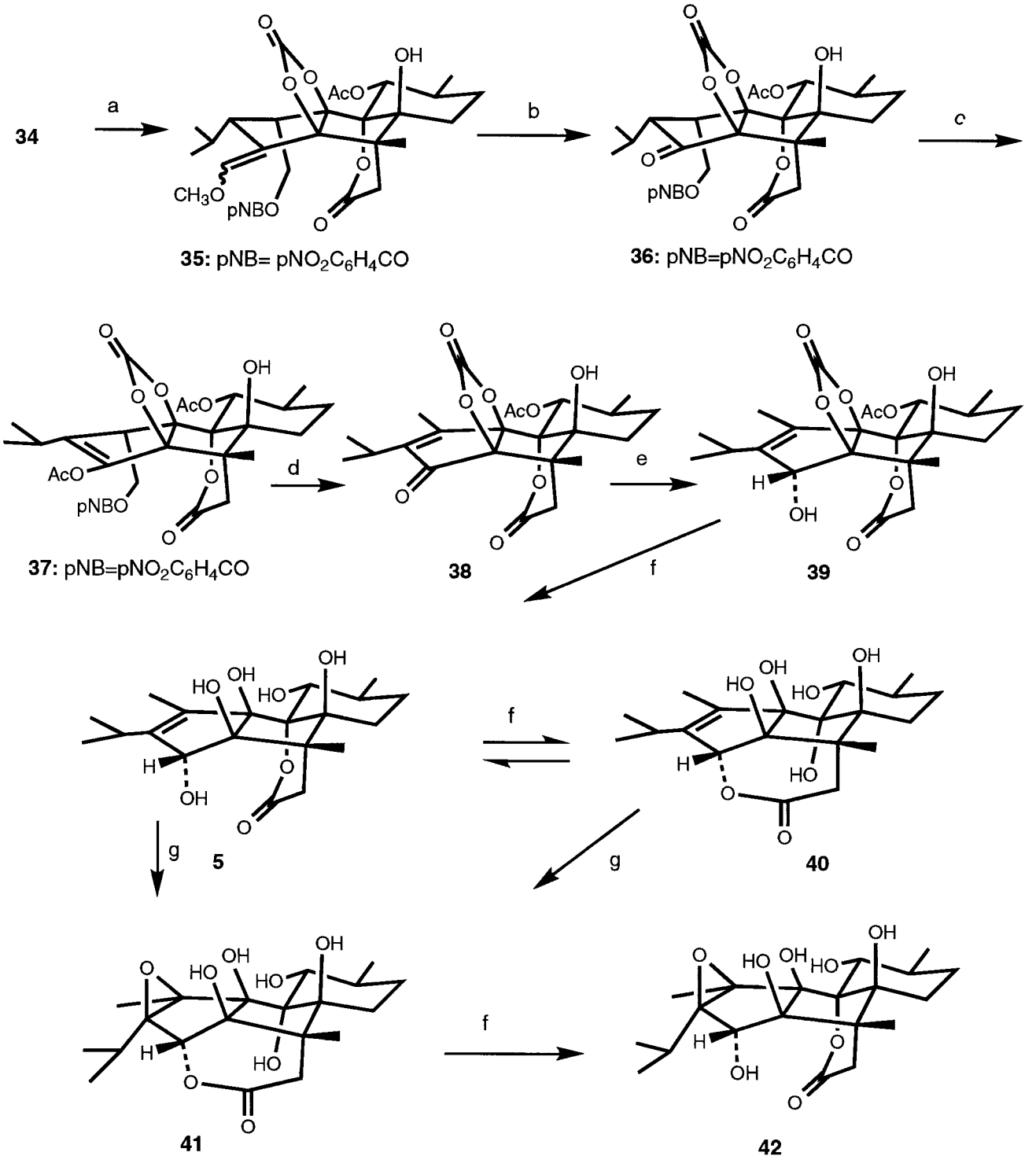

FIG. 8. Total synthesis of ryanodol: preparation of ring A.

sulted in 3-epiryanodol (**49**) in a good yield. Subsequent acylation (Neises and Steglich, 1978) of this compound with pyrrole-2-carboxylic acid gave 3-epiryanodine (**50**) with a satisfactory yield. The 3-epi isomer of naturally occurring ryanodine binds to the ryanodine receptor and provides the starting point for forming derivatives at the

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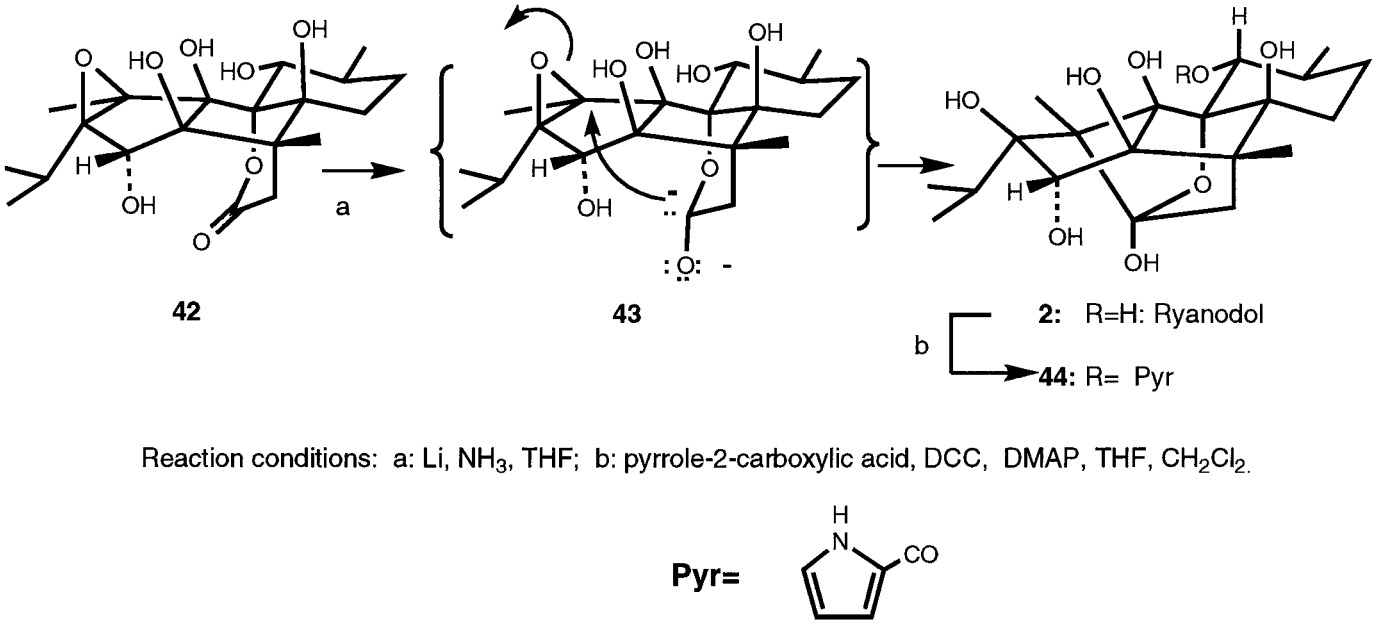

FIG. 9. Total synthesis of ryanodol: preparation of rings A and E.

C3 position (fig. 17, compounds **49A** through **49K**) (see section IV.I.2.).

D. 2-Deoxy-3-Epiryanodine Synthesis

Anhydroryanodol (**5**) was used very recently (Ruest and Dodier, 1996) as a starting material for the synthesis of 2-deoxy-3-epiryanodine (**53**). Selective oxidation (fig. 11) of the allylic hydroxyl gave enone **51**, which was treated with lithium in ammonia to yield crystalline 2-deoxy-3-epiryanodol (**52**) as the sole product; stereochemistry at C2 and C3 of this compound was established by x-ray diffraction (Drouin et al., 1996 and unpublished observations). Acylation (Yamada et al., 1974) of the 3-epi hydroxyl group with pyrrole-2-carboxylic acid and pyridine-3-carboxylic acid gave 2-deoxy-3-epiryanodine (**53**) and 2-deoxy-3-O-nicotinoyl-3-epiryanodol (**54**), respectively (Ruest and Dodier, 1996; Ruest et al., unpublished observations).

E. Ryanodine Analogs

1. Naturally occurring ryanoids, or ryanodine congeners. Since 1984, 9,21-dehydroryanodine (**55**) and several other naturally occurring ryanoids have been isolated from powdered *Ryania* stem wood (Waterhouse et al., 1984, 1985, 1987; Pessah et al., 1985; Ruest et al., 1985; Sutko et al., 1986; Humerickhouse et al., 1989, 1993; Jefferies et al., 1991, 1992a,b; Ruest and Dodier, 1996; Ruest et al., unpublished observations). Most of these compounds represent the ryanodine molecule at different states of oxidation and substitution. As shown in figure 12, they can be grouped in three main series (Jefferies et al., 1992a). The content of the extract may be directly appreciated by high performance liquid chromatography and nuclear magnetic resonance (NMR) methods. Depending on the country of origin of the wood, the content of some of the ryanoids, particularly the minor ones, can vary. The ryanodine preparations that have been available from Merck, Penick and Progressive Agri-Systems all contained two components in ratios that varied between different preparations from 30:70 to 70:30 (Ruest et al., 1985; Waterhouse et al., 1984, 1987; Jefferies et al., 1991, 1992a,b). The second component has been identified as 9,21-dehydroryanodine (Ruest et al., 1985; Jefferies et al., 1991, 1992b) and is similar to ryanodine in affinity for and in potency of its effects on the vertebrate RyRs. This compound accounts for a significant fraction of the biological activity in Ryania wood that was attributed initially to ryanodine alone. The presence of this major ryanoid probably was not observed originally because it is more soluble in ether than ryanodine. Ryanodine was initially purified by fractional crystallization from ether (Kelly et al., 1951). The discovery of 9,21-dehydroryanodine was important because, as described below in section IV.H., the 9,21 double bond provides a useful site for chemical derivatization.

The structures of the congeners of ryanodine (**55–71**) have been established by extensive ^{1}H NMR and ^{13}C NMR analyses (e.g., COSY, NOESY, and INVXCOR) (Waterhouse et al., 1984, 1985, 1987; Pessah et al., 1985; Jefferies et al., 1991, 1992a,b; Humerickhouse et al., 1993; Ruest and Dodier, 1996; Ruest et al., unpublished observations), by x-ray analysis (Ruest and Dodier, 1996; Ruest et al., unpublished observations) and by comparison with ryanodine. The methyl ether moiety in esters A (**65**) and D (**66**) initially located at position 4 (Ruest et al., 1985) has been shown recently to be situated at position 10 (epi) (Jefferies et al., 1992a). Also,

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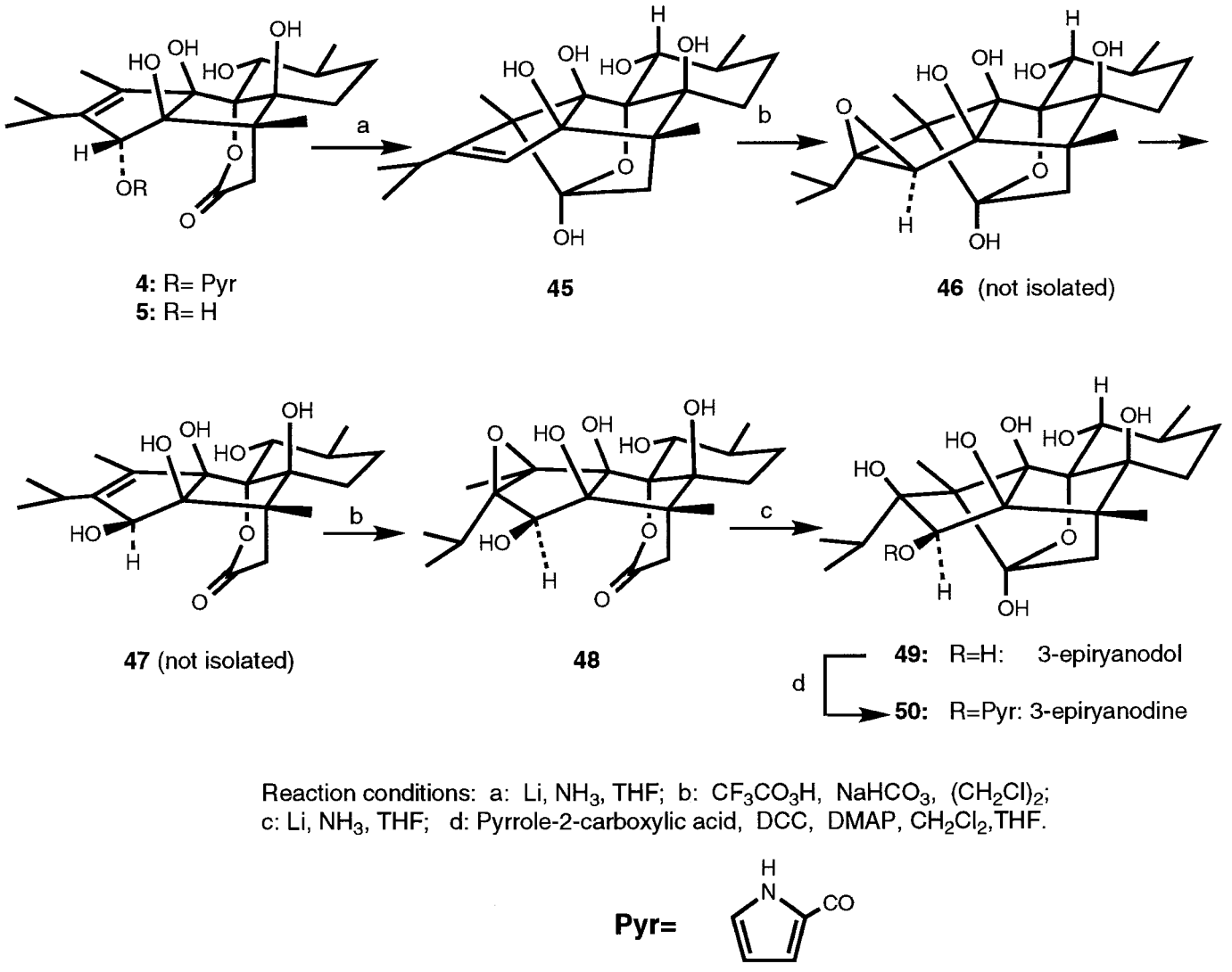

FIG. 10. Synthesis of 3-epiryanodine from anhydroryanodol or anhydroryanodine.

very recently, the hydroxyl moiety at the isopropyl group of a certain ryanoid **60**, formerly located at position 18 (Waterhouse et al., 1987), has been corrected and is located at position 19, based on evidence from x-ray diffraction performed on that compound (Ruest and Dodier, 1996). This deduction came after the isolation and identification of another ryanoid (**58**) that is different from one reported earlier (Waterhouse et al., 1987) and is hydroxylated at position 18, based on evidence from NMR analysis. Of special interest are compounds **63** and **64**, in which the pyrrole aromatic ring at position 3 has been exchanged for a pyridine and a benzene ring, respectively. NMR analysis has also permitted the conformations (syn or anti) adopted by the pyrrole carboxylate moiety in the ryanoids to be evaluated (Kaye et al., 1980; Jefferies et al., 1992a; Drouin et al., 1996 and unpublished observations). The conformation assumed can be altered by changing the solvent (Jefferies et al., 1992a).

The ryanodine congeners have been found to differ in their biological activities in interesting ways. For example, as described above, whereas ryanodine activates and inhibits the ryanodine receptor channel at submicromolar and micromolar concentrations, respectively, the ester A (**65**) only activates the channel at concentrations up to 3 mM (Sutko et al., 1990).

F. Ryanodine Derivatives: Chemical Modifications of Ryanodine

The ryanodine molecule has been derivatized extensively, first by Wiesner and coworkers to elucidate its structure (Wiesner, 1972) and subsequently by several groups, to establish structural features important for biological activity and to make experimentally useful analogs. The numerous chemical operations that yielded the different degradation products used by Wiesner (1972) will not be discussed here, as they have been described, rationalized and interpreted pre-

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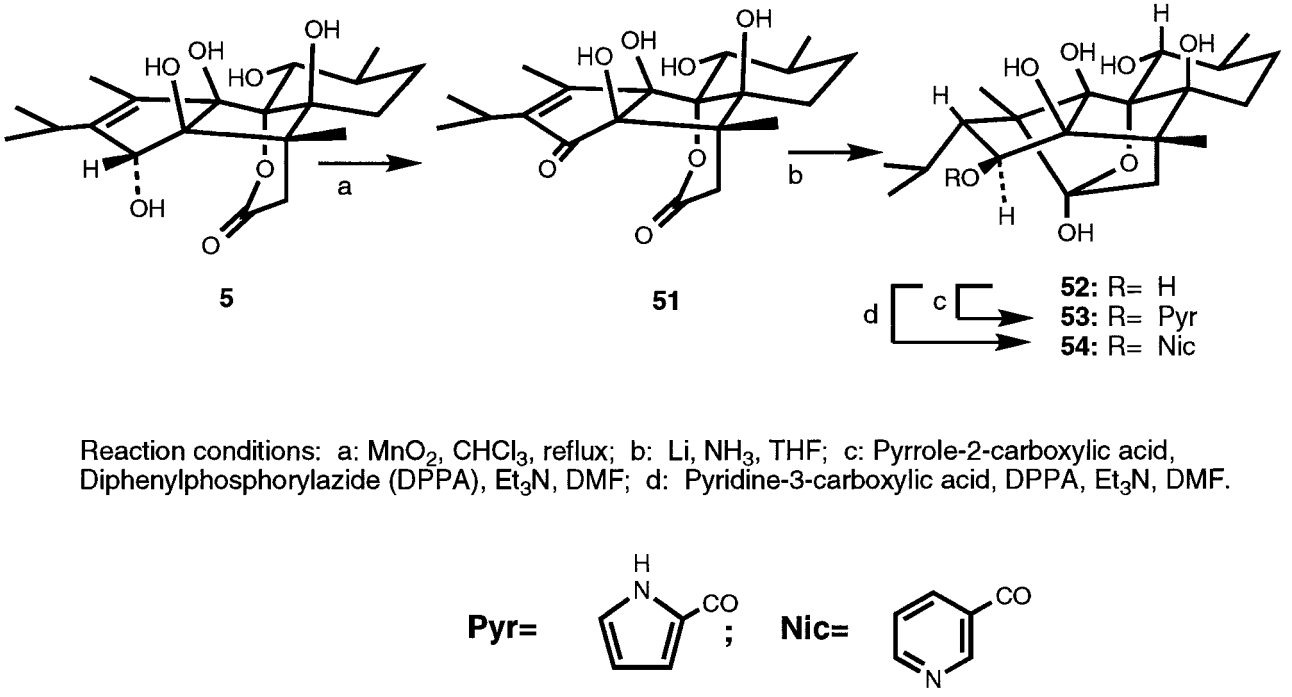

FIG. 11. Synthesis of 2-deoxy-3-epiryanodine from anhydroryanodol.

viously. The majority of the modifications made by other laboratories, including our own, have involved ryanodine (**1**), 9,21-dehydroryanodine (**55**) and ryanodol (**2**).

G. Modifications of Ryanodine

1. Tritium labeling of the pyrrole ring. Ryanodine was first tritiated by Fairhurst in 1971 (Fairhurst, 1971). The addition of tritium was achieved through aromatic bromination of the pyrrole ring followed by the catalytic reduction of the corresponding bromides with tritium gas. This method was improved at the bromination step by Waterhouse et al. (1987), using an acid scavenger in the reaction medium, and has been used to produce [^{3}H]ryanodine suitable for ligand binding studies (Pessah et al., 1985; Waterhouse et al., 1987).

In order to understand the importance of the polar substituents of the ryanodine molecule in its interactions with the RyR, several chemical transformations, including modification, blocking, and removal of these substituents, have been used.

2. O-alkylation and N-alkylation. O-alkylation and N-alkylation of ryanodine (**1**) (fig. 13) has been studied (Pessah et al., 1985; Waterhouse et al., 1987). Benzyl, *n*-butyl and methyl halides furnished the corresponding mono-substituted, di-substituted, tri-substituted, and tetra-substituted derivatives (see **72–77**). The pyrrole NH, and then the hemiketal hydroxyl at C15, were found to be the most reactive groups, followed by the tertiary hydroxyl at C4 and/or C6, and the secondary hydroxyl at C10. The corresponding structures were established using ^{1}H-NMR and ^{13}C-NMR spectroscopy. Very recently (Jefferies et al., 1996a,b), more specific methods of alkylating ryanodine have been used to obtain specific compounds. In general, the removal of the acidic hydrogens of the −OH or NH groups by alkylation, decreased the affinity with which the derivative bound to the RyR.

3. Selective O-acylation. Selective O-acylation of ryanodine has been studied at position 10 with several carboxylic acids (fig. 14) to give the corresponding ester derivatives such as acetate **78** (Pessah et al., 1985; Waterhouse et al., 1987), N-CBZ-2-aminoacetate **79** (Gerzon et al., 1993), N-CBZ-3-aminopropanoate **80**, N,N′-*bis*-CBZ-3-guanidino-propanoate **81**, and N,N′-*bis*-CBZ-2-guanidinoacetate **82** (Gerzon et al., 1993; Kahl et al., 1994). Reduction of the CBZ moieties by catalytic hydrogenation led to 2-aminoacetate **83** (glycyl), 3-aminopropanoate **84A** (β-alanyl), 2-guanidinoacetate **85**, and 3-guanidinopropanoate **86** (Gerzon et al., 1993). The amino group of compound **84A** also was acylated with 4-azidobenzoyl,2-nitro-5-azidobenzoyl and 4-benzoylbenzoyl moieties (see **84B-D**) (Kahl et al., 1994; Witcher et al., 1994). The hemisuccinate **87** and N-methylsuccinamidate **88** derivatives also have been synthesized (Gerzon et al., 1993). In addition, the C10-hydroxyl has been acylated by N-(m-iodobenzyloxycarbonyl)-β-alanine to give a nonradioactive derivative **89**, which was labeled subsequently with ^{125}I by isotope exchange (Mais et al., 1992). Some of the precedent basic esters have a greater affinity for the high affinity ryanodine binding site than ryanodine (Humerickhouse et al., 1993; Gerzon et al., 1993).

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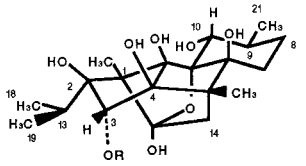
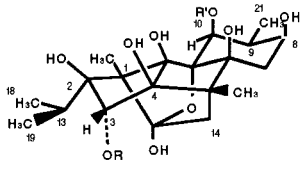
Ryanodine Series



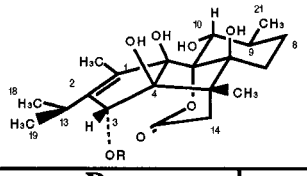
Designation	R	cont. (ppm, w/w)	other subst.	Ref.#
Ryanodine (**1**)	Pyr	450-600		a-c
9,21-Dehydroryanodine (**55**)	«	650-700		c-i
9,21α-Epoxyryanodine (**56**)	«	<10		j
9α-Hydroxyryanodine (**57**)	«	20-30	9_{ax}-OH	k
18-Hydroxyryanodine (**58**)	«	<10		j
18-Hydroxy-9,21-dehydroryanodine (**59**)	«	<10		j, l
19-Hydroxyryanodine (**60**)	«	<10		f, j
Ester B (**61**)	«	<10	$\Delta^{9,10}$; 8-oxo	i
Ester C_1 (**62**)	«	7-17	9_{ax}-OH, 10-epi	c, i
Ester G (3-O-Nicotinoylryanodol) (**63**)	Nic	42-58		c, m
18-Hydroxy-3-O-benzoylryanodol (**64**)	Bz	<10		j

8_{ax}-Hydroxy-10-epiryanodine series



Designation	R	R'	cont. ppm	other subst.	Ref.#
Ester A (**65**)	Pyr	CH_3	28-33		i, n
Ester D (**66**)	"	"	22-32	$\Delta^{9,21}$	i, n
Ester E (**67**)	"	"	<10	18-OH	c
Ester F (**68**)	"	H	30-64	$\Delta^{9,21}$	o, k

Anhydroryanodine series



Designation	R	cont. ppm	other subst.	Ref.#
Anhydroryanodine (**4**)	Pyr	<10		p
Anhydrodehydroryanodine (**69**)	"	<10	$\Delta^{9,21}$	p
Anhydro Ester A (**70**)	"	<10	8_{ax}-OH, 10epi-OCH_3	p
Ester C_2 (**71**)	"	12-17	9_{ax}-OH	c, g

Abbreviation: (ppm): content in parts per million; (w/w): weight of pure substance over weight of dry powder wood

Legend: Pyr=1H-Pyrrol-2-ylcarbonyl; Nic= Pyridin-3-ylcarbonyl; Bz=Benzoyl; Ref. #: a:Rogers et al., 1948; b: Wiesner, 1972; c: Jefferies et al., 1992b; d: Waterhouse et al., 1984; e: Waterhouse et al., 1985; f: Waterhouse et al., 1987; g: Pessah et al., 1985; h: Sutko et al., 1986; i: Ruest et al., 1985; j: Ruest and Dodier, 1996; k: Humerickhouse et al., 1993; l: Ruest et al., unpublished result; m: Jefferies et al., 1991; n: Jefferies et al., 1992a; o: Humerickhouse et al., 1989; p: Ruest and Deslongchamps, unpublished results.

FIG. 12. Natural ryanoids isolated from *Ryania speciosa Vahl*.

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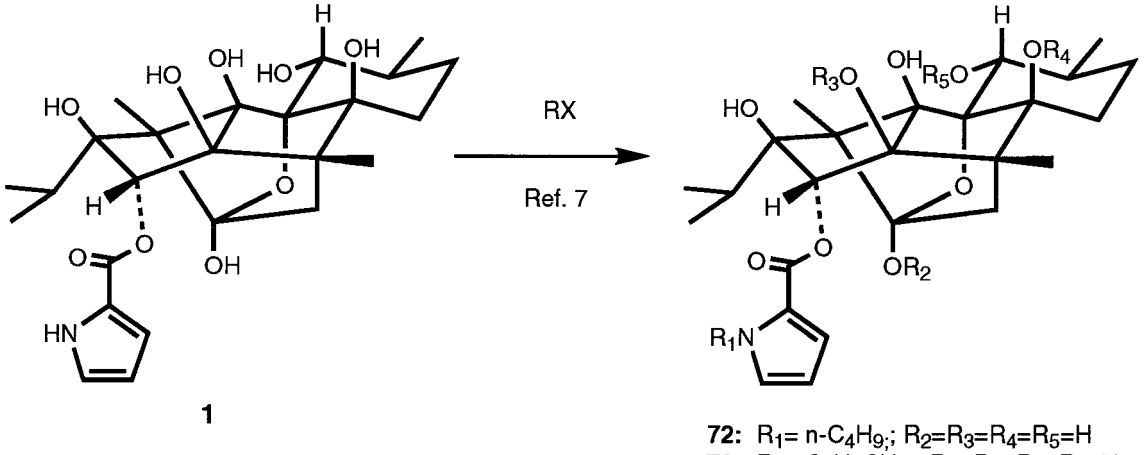

FIG. 13. Alkylation of ryanodine.

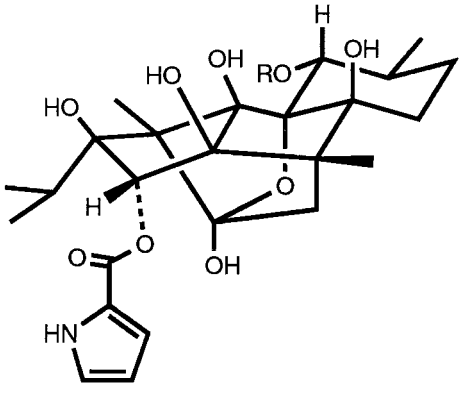

FIG. 14. Structures of ryanodine and some 10-O-acyl derivatives.

4. Selective oxidation. Selective oxidation of ryanodine (fig. 15) has been performed at position 10 to yield 10-oxoryanodine (**90**) and also in a degradative way by ozone in the pyrrole ring (Pessah et al., 1985; Waterhouse et al., 1987). The synthesis of several 10-oxoryanodine derivatives has been described very recently (Jefferies et al., 1996a) and is described at the end of this section (at section IV.I.4.).

5. Alterations at the C4 and C12 positions. Alterations at the C4 and C12 positions of ryanodine. Recently, Jefferies et al. (1993) used the periodate 4,12-*cis*-glycol oxidative cleavage used by Wiesner (1972) to prepare several derivatives of 4,12-seco-4,12-dioxoryanodine (**92**) (see fig. 16). These derivatives, obtained by the addition of several nucleophiles of different sizes and polarities at the C4 and C12 carbonyl groups, have been used to study the hydrophilic face of the molecule and to analyze their impact on the affinity of the derivative for binding to the RyR. These substituents, starting with simple hydrides (exoreduction of C4 and/or C12 carbonyl groups, leading to internal hemiketals, see **94**), diols (see **93**), hydroxyamine hemiketals (see **95**), oximes (see **108**, **109**), methyloximes, and benzyloximes to hydrazines, semicarbazines, and derivatives (**93-113**), involve markedly different structures and were used to define the stereochemical and polar properties of the site at which

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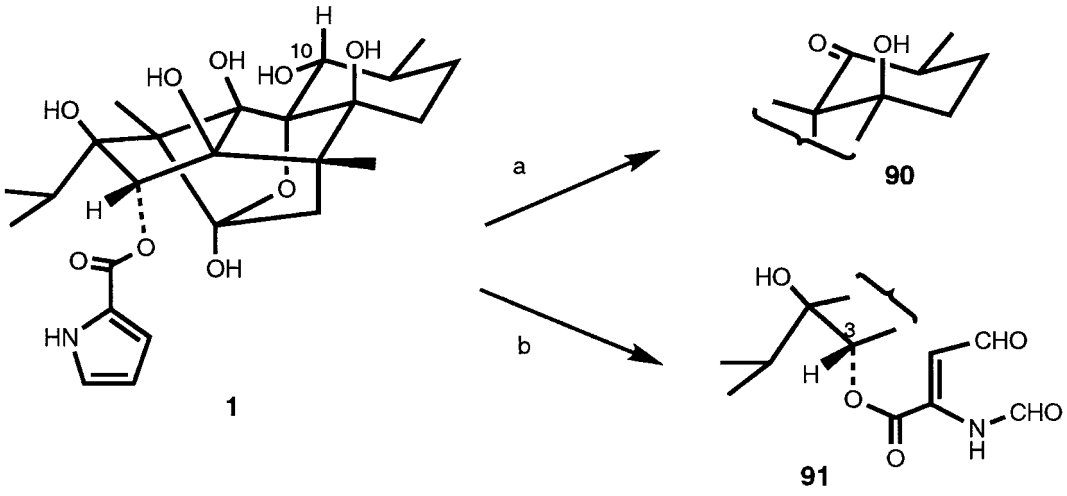

FIG. 15. Oxidation of ryanodine.

ryanodine binds to the RyR. The structure of each of these compounds was fully analyzed and characterized by a precise NMR analysis. It was concluded from this study that a hydrophilic group (−OH, −NHOH, or $-NHNH_2$) at C4 and a small group (e.g., −H), which was best at C12, yielded optimal interactions between the polar face of these ryanoids and the RyR (Jefferies et al., 1993).

6. Alterations at C3 and C2. Alterations at C3 and C2 of ryanodine. Synthesis of 3-epiryanodine (**50**, fig. 10) (Ruest and Deslongchamps, 1993) demonstrated the possibility of an overall "epimerization" at position 3 of ryanodine. Several derivatives in this series have been prepared (Ruest and Dodier, 1996) (see fig. 17, compounds **49A**-**49K**). The synthesis from anhydroryanodol (**5**) (fig. 11) of 2-deoxy-3-epiryanodine (**53**) that no longer has a hydroxyl at position 2 (fig. 11) (section IV.D.) also has demonstrated the possibility of preparing derivatives (see **54**) in this new series of compounds (Ruest and Dodier, 1996). Other modifications at the C2 and C13 positions have been published very recently (Jefferies et al., 1996a) (see section IV.I.3.).

H. Modifications of 9,21-Dehydroryanodine

The exocyclic double bond (C9-C21) in dehydroryanodine (**55**) has been used to introduce chemical alterations onto ring C of ryanodine.

1. Tritiation by catalytic hydrogenation. Tritiation by catalytic hydrogenation (Ruest et al., 1985; Pessah et al., 1985; Sutko et al., 1986; Waterhouse et al., 1987) of this double bond yields ryanodine (**1**, fig. 18) and 9-epiryanodine (**117**). Using this reaction, it was possible to introduce tritium at these positions, yielding tritiated forms of ryanodine and 9-epiryanodine with specific activities (60.8 and 45.8 Ci/mmole, respectively) that are suitable for use in radioligand binding studies (Sutko et al., 1986). This method is used currently to produce commercially available [^{3}H]ryanodine. The availability of isotopically labeled ryanodine has permitted the identification and purification of the ryanodine receptor protein, and, ultimately, the cloning and sequencing of its messenger ribonucleic acid.

2. Oxidation of 9,21-dehydroryanodine. 8,9-dehydro-10-oxoryanodine (**118**) can be obtained directly by oxidation of 9,21-dehydroryanodine (**55**) (fig. 18) (Waterhouse et al., 1987).

3. 21-thioether adducts and derivatives. Mercaptoethanol and 4-mercaptobutanol react readily and stereospecifically with 9,21-dehydroryanodine (fig. 16) to give the corresponding adducts **114A** and **114D** (Kahl et al., 1994). These thioether derivatives have been transformed to their 4-azidobenzoyl derivatives **114B** and **114E** and also to a 3,3,3-trifluoro-2-diazopropionyl derivative **114C** (Kahl et al., 1994). Synthesis of photolabile cross-linking azido derivatives has been useful for defining the sites of interactions between ryanoids and RyRs. Propanethiol and t-butylthiol also add to C21 (AIBN) to give the corresponding thioethers (see **115** and **116**) (Jefferies et al., 1993).

4. Epoxidation and dihydroxylation of the 9,21 double bond. As shown in fig. 18, epoxidation of the 9,21 double bond has led to 9,21α-epoxide (which also have been obtained as a natural ryanoid, see **56**, fig. 12) and 9,21β-epoxide (see **119** and **120**, fig. 18) (Pessah et al., 1985; Waterhouse et al., 1987; Welch et al., 1996a). Dihydroxylation of this double bond gave 9,21-dihydroxy-9-epiryanodine (**121A**, R = H) (Pessah et al., 1985; Waterhouse et al., 1987; Kahl et al., 1994). The 21-hydroxyl group of the latter compound also has been acylated by the 4-azidobenzoyl moiety to give derivative **121B**, which

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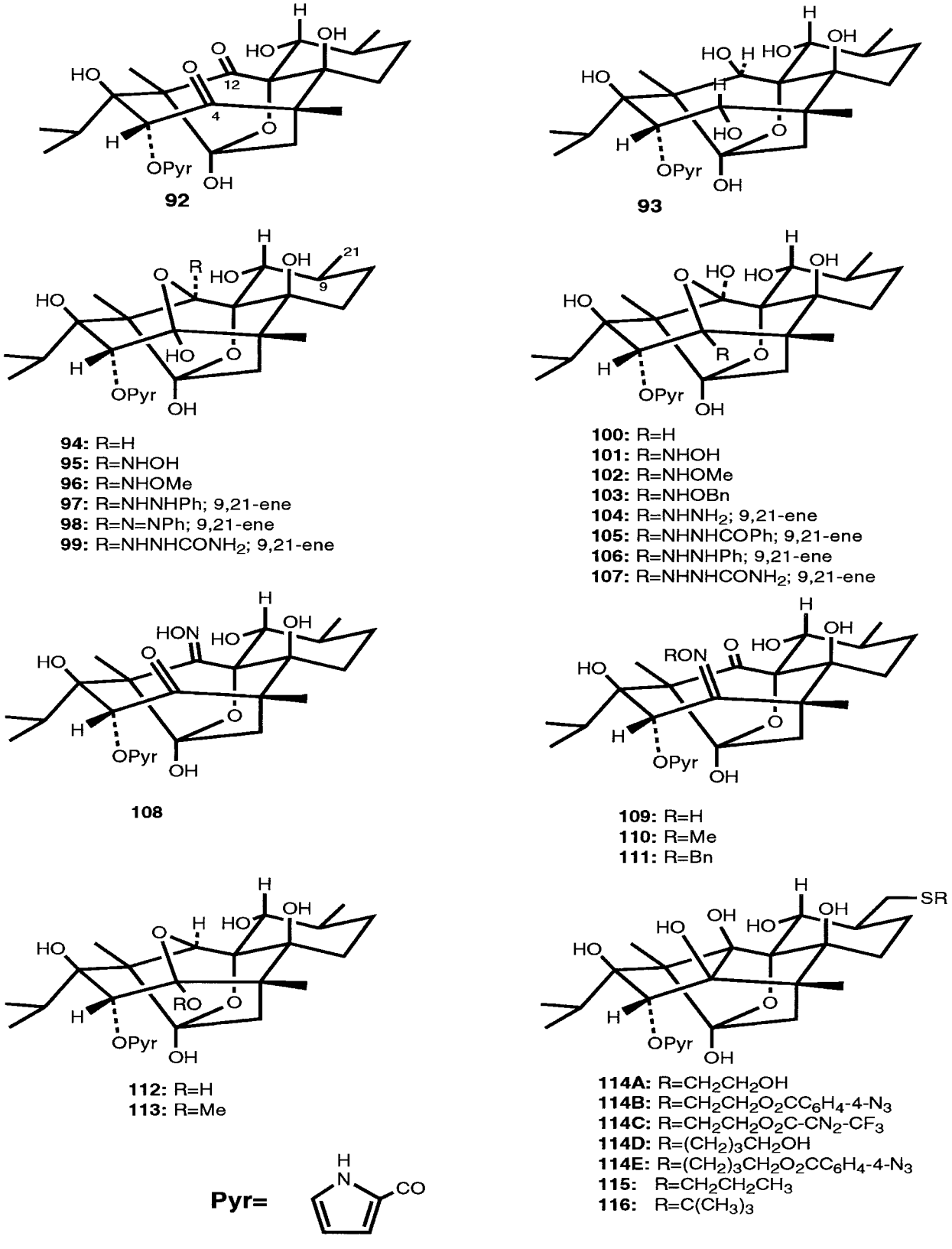

FIG. 16. Structures of ryanodine, 9,21-dehydroryanodine and some derivatives at positions 4, 12 and 21.

has been used to characterize the ryanodine binding site (Kahl et al., 1994). Alternatively, 9,21α-epoxyryanodine (**119**) was used to introduce nitrogen containing (azido and amino) groups at position 21 while leaving an axial hydroxyl group at C9 (see **122** and **123**) (Welch et al., 1996a). Some 21-N-acyl derivatives also have been pre-

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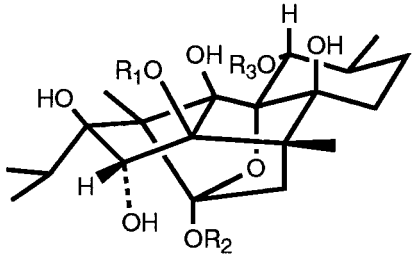
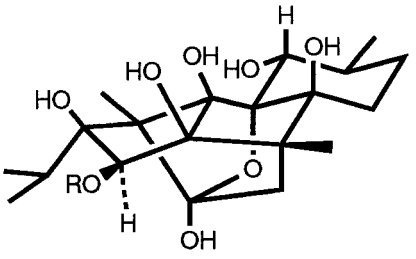


2: $R_1=R_2=R_3=$ H: Ryanodol
2A: $R_1=R_2=$H; $R_3=$ $CBZNHCH_2CO$-
2B: $R_1=R_2=$H; $R_3=$ $CBZNHCH_2CH_2CO$-
2C: $R_1=R_2=$H; $R_3=$ NH_2CH_2CO-
2D: $R_1=R_2=$H; $R_3=$ $NH_2CH_2CH_2CO$-
2E: $R_1=R_2=C_6H_5CH_2$-; $R_3=$ H
2F: $R_1=R_2=C_6H_5CH_2$-; $R_3=$ CH_3CO-
2G: $R_1=R_2=C_6H_5CH_2$-; $R_3=$ C_6H_5CO-
2H: $R_1=R_2=$H; $R_3=$ CH_3CO-
2I: $R_1=R_2=$H; $R_3=$ C_6H_5CO-
2J: $R_1=R_2=$H; $R_3=$ Pyridine-3-carbonyl-



49: R=H: 3-Epiryanodol
49A: R= CH_3CO-
49B: R= C_6H_5CO-
49C: R= Pyridine-3-carbonyl-
49D: R= Indole-2-carbonyl-
49F: R= Indole-3-carbonyl-
49G: R= $CBZNHCH_2CO$-
49H: R= NH_2CH_2CO-
49I: R= $CBZNHCH_2CH_2CO$-
49J: R= Thiophene-2-carbonyl-
49K: R= Furane-2-carbonyl-

(CBZ= $C_6H_5CH_2OC(O)$-)

FIG. 17. Structures of ryanodol, 3-epiryanodol and some derivatives.

pared (see **124** and **125**), the latter being a fluorescent derivative (Welch et al., 1996a,b). Some very recently accomplished modifications in the cyclohexane ring (Jefferies et al., 1996b) are described at the end of this section.

5. 8,9-Dehydroryanodine. Isomerization (fig. 19) of the exocyclic double bond of 9,21-dehydroryanodine (**55**) with activated palladium on charcoal in hot xylene quantitatively produced 8,9-dehydroryanodine (**126**), creating access for additional modification at position 8 (Ruest and Deslongchamps, unpublished observations; Welch et al., 1997).

6. 8-Amino-9-hydroxyryanodine. Epoxidation of 8,9-dehydroryanodine (**126**) gave separable α-epoxides and β-epoxides (**127** and **128**) (fig. 19) that were regioselectively opened to the corresponding 8-azido-9-hydroxyryanodines (**129** and **130**). Reduction of the latter compounds yielded the aminoalcohol derivatives **131** and **132** (Ruest and Deslongchamps, unpublished observations; Welch et al., 1997).

7. 4,12-Seco-4,12-dioxo-9,21-dehydroryanodine and derivatives. As described above for ryanodine, modifications in this series were performed in parallel with 9,21-dehydroryanodine (fig. 16, see compounds **97**–**99** and **104**–**107**) to investigate their impact on the binding of the derivatives to the RyR (Jefferies et al., 1993).

I. Modifications of Ryanodol

1. 10-O-Acylryanodol. Several acyl derivatives of ryanodol (**2**) have been prepared recently. These compounds (see **2A** to **2J**, fig. 17) were obtained mostly by direct esterification of ryanodol with the corresponding carboxylic acids (Neises and Steglich, 1978). This yielded 10-O-pyrrolecarbonylryanodol (**50**) (see fig. 10) (Ruest and Deslongchamps, 1993), 10-O-(N-CBZ-2-aminoacetyl)ryanodol (**2A**), and 10-O-(N-CBZ-3-aminopropanoyl)ryanodol (**2B**) (Ruest and Deslongchamps, unpublished observations; Ruest et al., unpublished observations). The latter two derivatives furnished the corresponding aminoester derivatives **2C** and **2D** upon hydrogenolysis of the protecting group (Ruest and Deslongchamps, unpublished observations; Ruest et al., unpublished observations). Some other 10-O-acyl derivatives (see **2H**, **2I**) of ryanodol were accessible only through its 4,15-di-O-benzyl ether **2E** (Deslongchamps et al., 1990), which was acylated with acetic anhydride or benzoyl chloride to give **2F** and **2G**, followed by catalytic hydrogenolysis of the protecting groups, giving **2H** and **2I**, respectively. It has been possible to obtain the 10-O-nicotinoyl derivative (**2J**) by simple acylation of ryanodol with nicotinoyl chloride hydrochloride in triethylamine (Ruest et al., unpublished observations; Ruest and Dodier, unpublished observations).

2. 3-O-Acyl-3-epiryanodol. Acylation of the 3-epi-hydroxyl group was shown to be particularly easy compared with its unreactive endo-epimer as it exists in ryanodol. Besides 3-epiryanodine (**50**) (fig. 10) described above (Ruest and Deslongchamps, 1993), the following derivatives were prepared by O-acylation with the corresponding acids (fig. 17) (Neises and Steglich, 1978): acetyl (**49A**), benzoyl (**49B**), nicotinoyl (**49C**), indole-2-carbonyl (**49D**), indole-3-carbonyl (**49F**), N-CBZ-glycyl (**49G**) and glycyl (**49H**), N-CBZ-β-alanyl (**49I**), thiophene-2-carbonyl (**49J**), and furane-2-carbonyl (**49K**)

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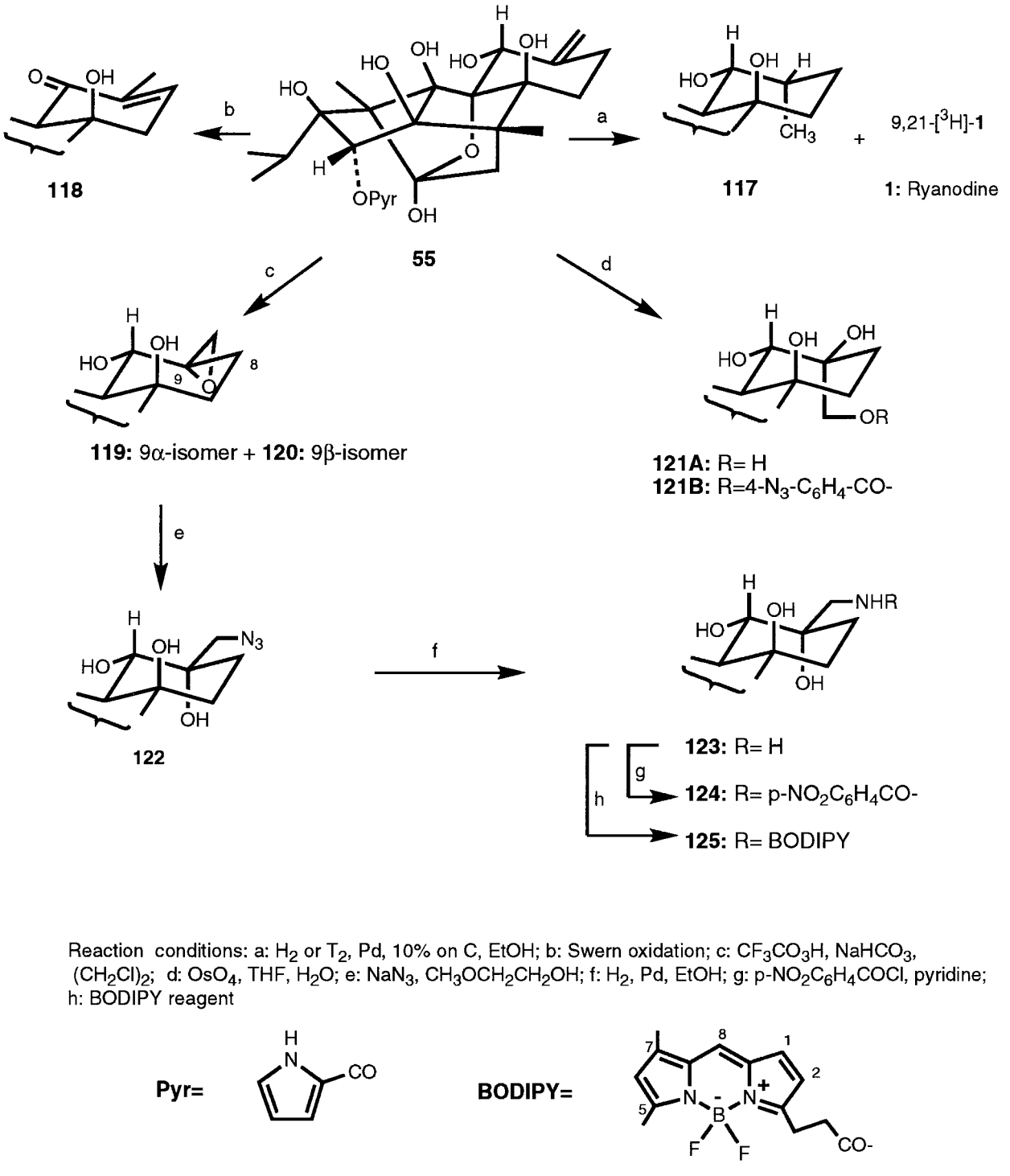

FIG. 18. Derivatization of 9,21-dehydroryanodine at positions, 9, 10 and 21.

(Ruest and Dodier, unpublished observations; Ruest et al., unpublished observations).

3. 3-Deoxyryanodol (cinnzeylanol) (**6A**). In order to measure the importance of polar groups in ring A, the hydroxyl groups at C3 and C2 of ryanodol were removed in the following way (fig. 20) (Ruest and Dodier, 1996): 2,3-dideoxy-2,3-dehydroryanodol (**45**) was subjected to acidic medium and yielded 3-deoxyanhydroryanodol (**133**). Upon oxidation to epoxide **134** followed by treatment with lithium in ammonia, this compound gave crystalline cinnzeylanol (**6A**), the structure of which was confirmed by x-ray diffraction (Drouin et al., 1996 and unpublished observations). Acetylation of 3-deoxyryanodol (**6A**) yielded cinnzeylanine (10-O-acetyl-3-deoxyryanodol) (**6B**) (Ruest and Dodier, 1996). These last experiments represent the first known synthesis of these two natural insecticidal compounds isolated from *Cinnamomi cortex* by Nohara et al. (1981). Alternatively, 3-de-

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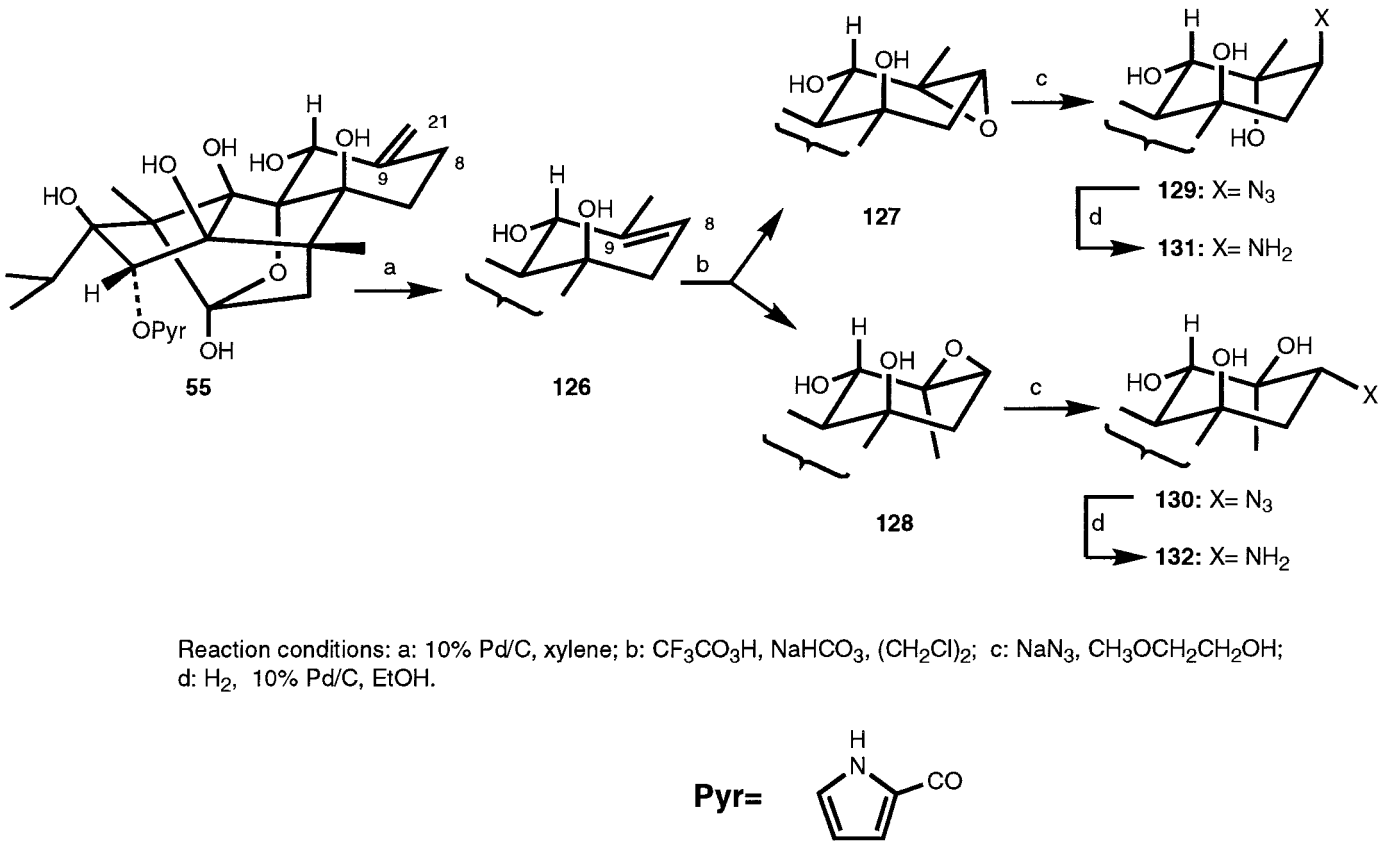

FIG. 19. Formation of 8-amino-9-hydroxyryanodine from 9,21-dehydroryanodine.

oxyanhydroryanodol (**133**) was subjected directly to reductive cyclization by lithium in ammonia, which yielded crystalline 2,3-dideoxy-2,3-dihydroryanodol (**135**). Stereochemistry at position 2 was established by x-ray diffraction (Ruest and Dodier, 1996; Drouin et al., 1996 and unpublished observations).

As this review was being finished, there were two additional reports (Jefferies et al., 1996a,b) of new aspects of ryanodine chemistry and of selective methods for derivatizing ryanoids. The importance of several of the hydroxyl groups in ryanodine and 9,21-dehydroryanodine has been examined for their importance to the biological activities of these compounds. The hydroxyls at the C4 and C6 positions (fig. 21) were blocked as cyclic borates and boronates (see **136** to **140**), and the hydroxyls at the C10 and C12 positions were blocked as cyclic phosphates, phosphonates, and phosphoramidates (see **141** to **147**). The cyclic borates were also shown to be useful for the preparation of selective acylated compounds (Jefferies et al., 1996b), as they can also be used as protecting groups, easily removed by slow distillation of methanol or treatment with methylamine. Dehydration (fig. 22) of the C2 hydroxyl giving 2-deoxy-2 (13)-dehydroryanodine (**148**) was accomplished using a modification of a known method (Wiesner, 1972). This olefin was epoxidized to compounds **149**α and **149**β and also dihydroxylated to 13-hydroxyryanodine (**150**) with osmium tetroxide. In general, these modifications resulted in a decreased affinity for binding to the RyR, with the exception of the boron derivatives. The latter may not be stable under the conditions used to assay their binding activities and undergo dissociation (Jefferies et al., 1996a).

4. New modifications in ring C of ryanodine and 9,21-dehydroryanodine (Jefferies et al., 1996b). A variety of derivatives of 10-oxo-ryanodine (**90**) have been prepared, including oximes, hydroxyamines, 10-epi-amino, 10-epi-hydroxy-, hydrazone, hydrazine, lactam and several additional derivatives of the preceding compounds (see fig. 23, compounds **151** to **162**, **171**), including a 10-epi-(4-azidobenzoyl)hydrazide (**162**) as a potential photoaffinity probe for labeling the RyR. Moreover, defunctionalization by classical methods of the cyclohexane ring of 9,21-dehydroryanodine (**55**) led to the 21-nor-9-oxo compound (**163**), which provided access to the 9_{ax}- and 9_{eq}-hydroxy derivatives (**164**, **165**) and to 21-nor-10-deoxyryanodine (**166**). This compound was then transformed into 21-nor-10-deoxyryanodine (**167**), 10-deoxy-9,21-dehydroryanodine (**168**), and epimeric 10-deoxy-9-hydroxy compounds (**169**, **170**). Five of these compounds exhibited significant affinities for binding to the RyR. These include, 10-epiryanodine (**153**), 10-deoxy-10-amino-epiryanodine and methoxyamino-10-epiryanodine (**154**, **158**), the 10-epi-azidobenzoylhydrazide derivative (**162**), and 10-deoxy-9,21-dehydroryanodine (**168**). Together with the chemical approaches described above

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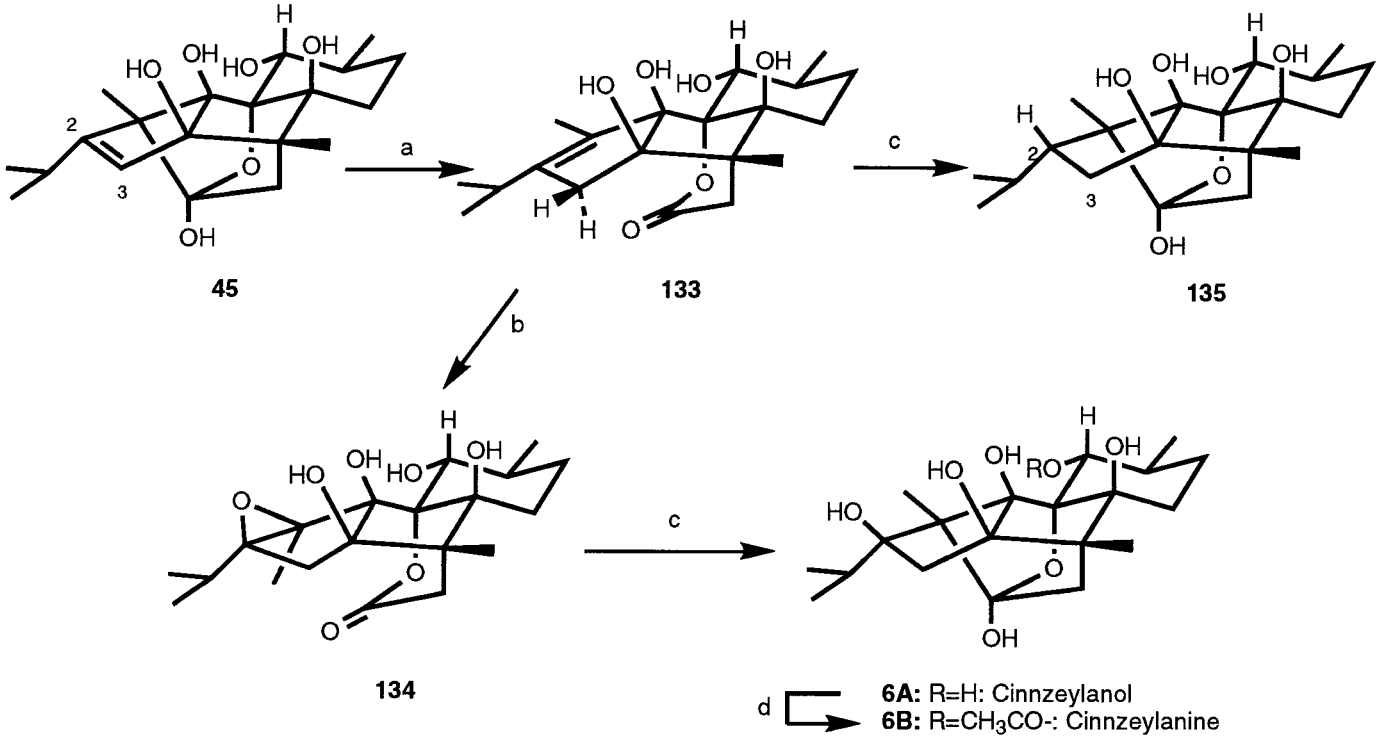

FIG. 20. Synthesis of cinnzeylanol (3-deoxyryanodol) and cinnzeylanine from anhydroryanodol.

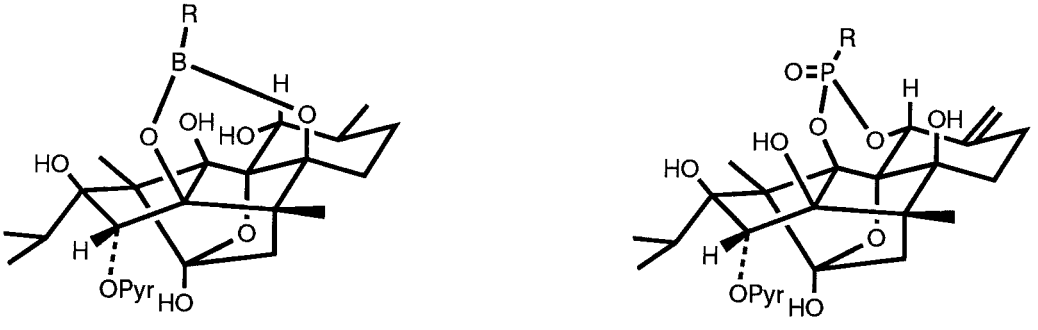

FIG. 21. Structures of ryanodine, 9,21-dehydroryanodine and some 46-boronate and 10,12-phosphonate derivatives.

(from sections IV.G. to IV.I.3.), these recently developed methods provide a broad access to the chemistry of ring C of the ryanoids. The binding activities of the agents described in this section that are known are summarized in table 1.

In summary, as noted previously, the motivation for investigating many of these chemical modifications has been to establish the properties of the ryanodine molecule that are important for binding to the RyRs and for producing changes in the channel function of these pro-

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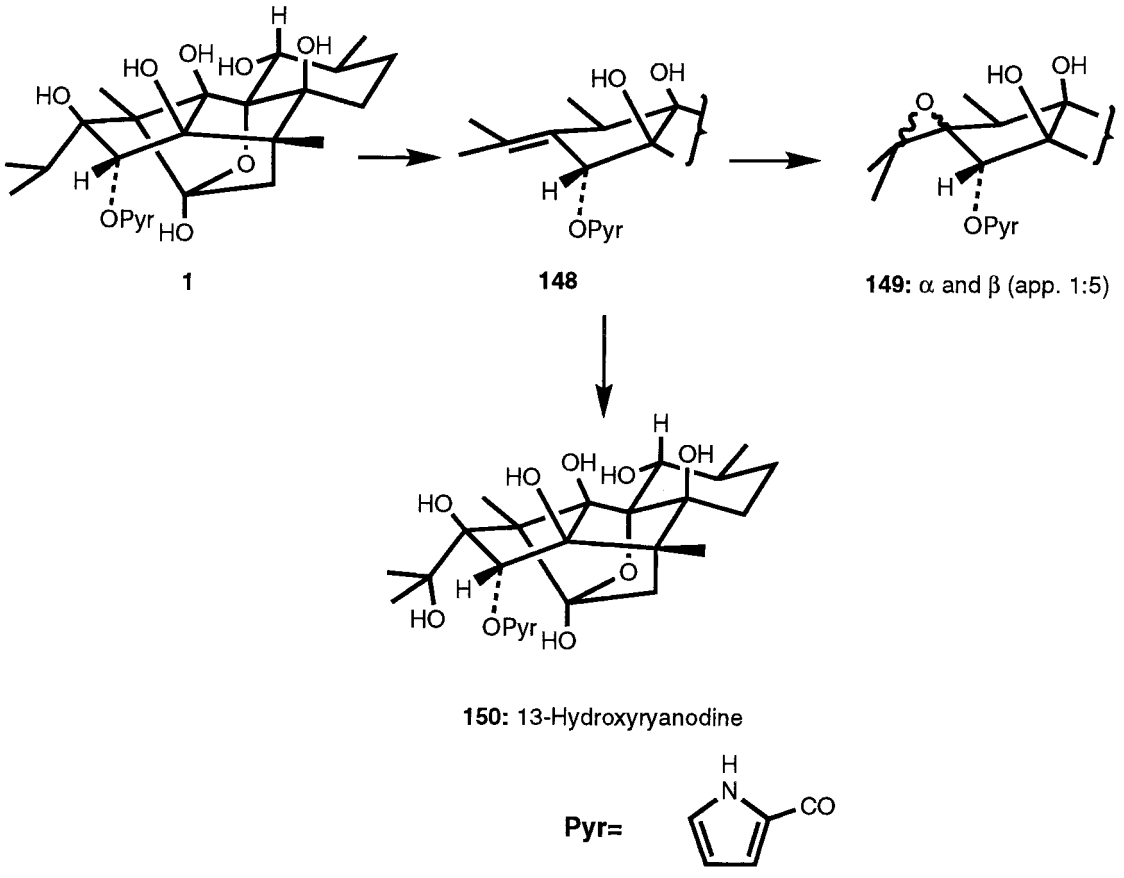

FIG. 22. Synthesis of 13-hydroxyryanodine from ryanodine.

teins. The identification of naturally occurring ryanodine congeners and the selective derivatization of specific aspects of the ryanodine molecule have permitted new insights into these properties. These results and those obtained from molecular modeling of the ability of ryanoids to bind with high affinity to RyRs and to alter the conductance of RyR channels are discussed further in section V.

V. Ryanoids: Structure/Function Relationships

A. Biological Relevance

Investigations of the relationships between the structure of ryanodine (**1**) and the ability to bind to the RyRs with high affinity and to modify the channel properties of these proteins have several goals. These are as follows:

- to identify the structural features of the ryanodine molecule that serve as determinants for high affinity binding and for alterations of the properties of the RyR channel. This information will permit the design of compounds that bind specifically to different RyR isoforms and either activate or inhibit the RyR channels.
- to establish the minimal chemical structures that are required for these biological activities. This information will facilitate the synthesis of new compounds. To date, membrane proteins have largely resisted attempts to produce the crystals required for x-ray diffraction analyses of their three-dimensional structures. Thus, it is unlikely that high resolution structural information will be available for the RyRs in the near future. Consequently, a third objective is. . .
- to develop a complimentary picture of the physical properties of the ryanodine binding domains that exist in the different RyR isoforms. This work will yield insights into the topological features of the ryanodine binding domains in these proteins and will promote the development of isoform-specific compounds. The ability to produce dramatic changes in RyR channel function by interacting with the ryanodine binding site(s) implies the existence of endogenous effectors. At present, native compounds that interact with the ryanodine binding site of the RyRs have not been identified. Therefore, a fourth goal is. . .
- to obtain clues about the chemical identity of potential endogenous effectors of RyR function that act at the ryanodine binding site.

Achieving the preceding goals will permit the design of experimentally and perhaps clinically useful compounds that affect specific RyR isoforms and that selec-

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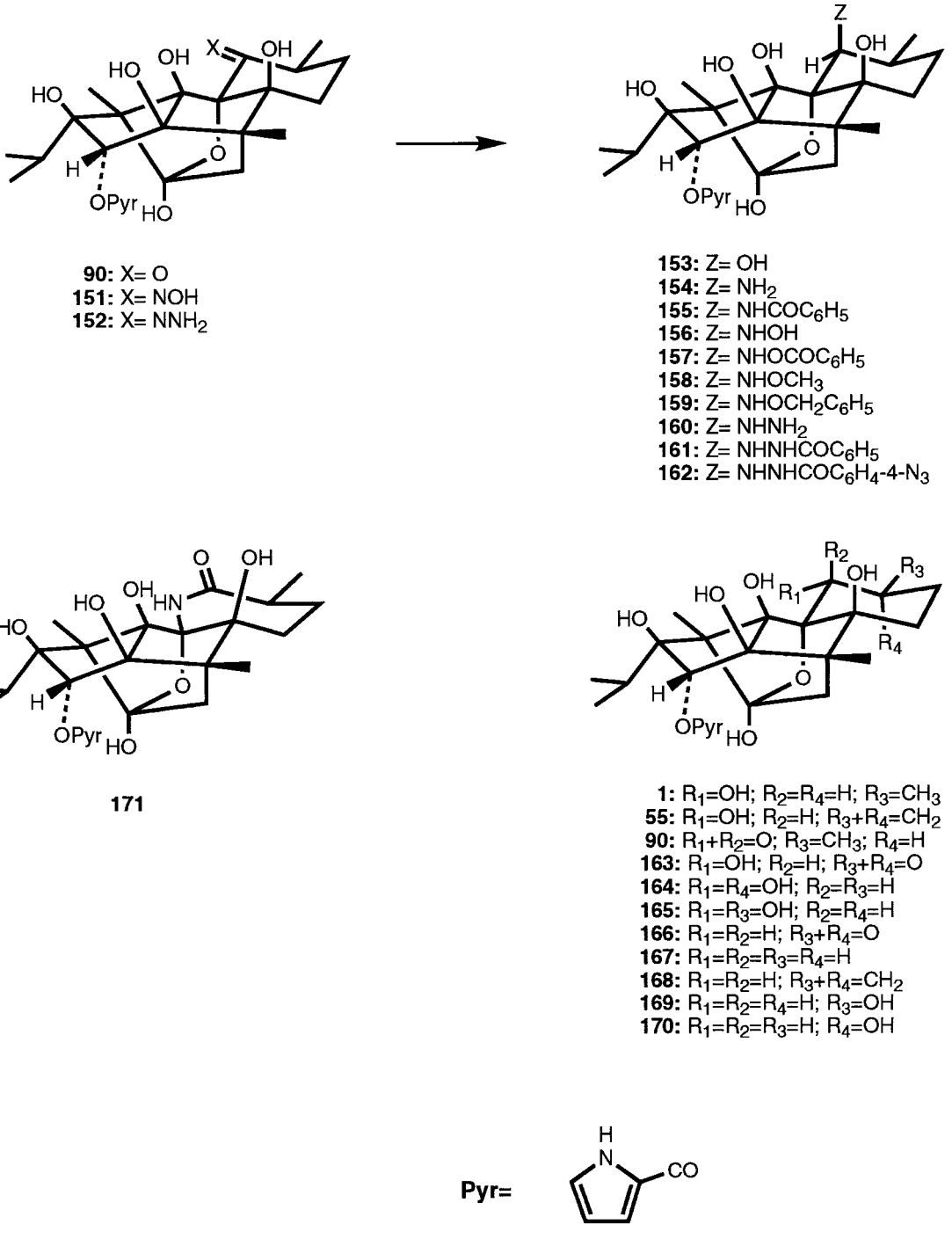

FIG. 23. Structures of ryanodine and derivatives modified in ring C.

tively activate or inhibit the RyR channel. As described above in section III, these compounds are required for defining how each of the RyR isoforms participates in Ca^{2+} signaling. These goals could be achieved in the time-proven way (by serendipity) or with malice of forethought by molecular design. While waiting for the former to strike, we have initiated studies using molecular modeling to investigate the structure-activity relationships of ryanodine (**1**) and related molecules (Tables 1, 2).

B. Molecular Modeling

The ability to apply sophisticated molecular modeling techniques has become much more widely achievable with the dramatic increase in computer power-to-price ratio that has occurred over the past decade. The use of a computational approach should be especially useful in the case of ryanodine (**1**), because little is known about the structure of the RyR binding site. The availability of a large number of ryanodine congeners and deriv-

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TABLE 1
Biological activities of ryanodine and related compounds

Scheme Number	Compound	Skeletal IC_{50} nM	Cardiac IC_{50} nM	Skeletal K_D nM	Cardiac K_D nM	Fractional Conductance
1	ryanodine	10[a]	2.8[bm]	7[k]	2.5[bo]	56.8[c]
2	ryanodol	35,000[a]	1725[bm]	12000[k]	1556[bo]	69.4[c]
2C	10-O-aminoacetyl-ryanodol		140[f]		346[b]	
4	anhydroryanodine	10,000[a]		2900[k]		
44	10-O-pyrrole-carbonyl-ryanodol		227[bm]	52[p]	13[o]	52.2[c]
50	3-epiryanodine		178[bm]	350[p]	178[bo]	42.9[c]
55	9,12-didehydroryanodine	13[a]	3.9[bm]	7[k]	2.5[bo]	58.3[c]
57	9α-hydroxyryanodine			110[k]		
60	19-hydroxyryanodine	88[a]				
61	ester B			240[k]		
62	ester C_1	500[d]		210[k]		
63	3-O-nicotinoyl-ryanodol	1429[d]	446[bm]	1100[k]	337[b]	
65	ester A	37.04[d]		110[k]	61[o]	61.5[c]
66	ester D	90.91[d]		51[k]		
67	ester E	>2000[d]				
68	ester F	556[d]				
70	anhydroester A			5200[k]		
71	9-hydroxy-anhydroryanodine	667[d]		2600[k]		
72	N-butyl-ryanodine	880[a]				
73	N-benzyl-ryanodine	310[a]				
74	N,15-O-dimethyl-ryanodine	280[a]				
75	N,4-O,15-O-trimethyl-ryanodine	4500[a]				
76	N,6,O,15-O-trimethyl-ryanodine	1300[a]				
77	N,4-O,10-O,15-O-tetramethyl-ryanodine	>10,000[a]				
78	10-acetyl-ryanodine	140[a]				
79	10-O-(NCBZ-aminoacetyl)-ryanodine	4.2[e]	62[bm]	1.4[e]	29[o]	29.4[c]
80	10-O-(NCBZ-3-aminopropanoyl)-ryanodine	6.4[e]		2.2[e]		
81	10-O-(N,N′-bis-CBZ-3-guanidinopropanoyl)-ryanodine	51.8[e]		20.4[e]		
82	10-O-(N,N′-bis-CBZ-2-guanidinoacetate)-ryanodine	33.2[e]		12.9[e]		
83	10-O-(2-aminoacetyl)-ryanodine	2.0[e]		0.5[e]		
84A	10-O-(3-aminopropanoyl)-ryanodine	1.5[e]	14.2[bm]	0.5[e]	0.61[o]	14.3[e]
84B	10-O-(3-[4-azidobenzamido]-propanoyl)-ryanodine	3.2[f]				
84C	10-O-(3-[2-nitro-5-azidobenzamido]-propanoyl)-ryanodine	36.6[e]		21.6[e]		
84D	10-O-(3-[4-benzoylbenzamido]-propanoyl)-ryanodine	6.0[f]				
85	10-O-(2-guanidinoacetyl)-ryanodine	1.3[e]		0.4[e]		
86	10-O-(guanidinopropanoyl)-ryanodine	1.4[e]	0.6[bm]	0.3[e]	0.55[bo]	5.8[c]
87	10-O-(hemisuccinyl)-ryanodine	>1000[g]				
88	10-O-(N-methylsuccinamidyl)-ryanodine	49.4[g]				
90	10-oxoryanodine	11.67[h]				
92	4,12-seco-4,12-dioxoryanodine	750[i]				
93	4,12-seco-4,12-dihydroryanodine	255[i]				
94	4,12-hemiketal-4-hydroxyryanodine	19.35[i]				
95	4,12-hemiketal-4-hydroxy-12-hydroxyamino-ryanodine	133[i]				
97	4,12-hemiketal-4-hydroxy-12-phenylhydrazino-ryanodine	461[i]				
98	4,12-hemiketal-4-hydroxy-12-phenylazo-ryanodine	255[i]				
99	4,12-hemiketal-4-hydroxy-12-semicarbazino-ryanodine	196[i]				
100	4,12-hemiketal-12-hydroxyryanodine	42.85[i]				
101	4,12-hemiketal-4-hydroxyamino-12-hydroxyryanodine	85.17[i]				
102	4,12-hemiketal-4-methoxyamino-12-hydroxyryanodine	52.17[i]				
103	4,12-hemiketal-4-benzyloxyamino-12-hydroxyryanodine	92.31[i]				
104	4,12-hemiketal-4-hydrazino-12-hydroxyryanodine	109[i]				
105	4,12-hemiketal-4-benzoylhydrazino-12-hydroxyryanodine	200[j]				
106	4,12-hemiketal-4-phenylhydrazino-12-hydroxyryanodine	222[i]				
107	4,12-hemiketal-4-semicarbazino-12-hydroxyryanodine	120[i]				
108	4,12-seco-4-oxo-12-hydroxyimino-ryanodine	705[i]				
109	4,12-seco-4-hydroxyimino-12-oxo-ryanodine	1579[i]				
110	4,12-seco-4-methoxyimino-12-oxo-ryanodine	1600[i]				
111	4,12-seco-4-benzyloxyimino-12-oxo-ryanodine	923[i]				
114A	21-(2-hydroxyethylmercapto)-ryanodine	>1200[j]				
114C	21-(2-[3,3,3-trifluoro-2-diazo-propanoyloxy]-ethylmercapto)-ryanodine	4[f]				
114D	21-(4-hydroxybutylmercapto)-ryanodine	25[f]				
114E	21-(4-[4-azidobenzoyloxy]-butylmercapto)-ryanodine	198[f]				

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TABLE 1
Continued

Scheme Number	Compound	Skeletal IC_{50} nM	Cardiac IC_{50} nM	Skeletal K_D nM	Cardiac K_D nM	Fractional Conductance
115	21-(propylmercapto)-ryanodine	>1200[j]				
116	21-(*t*-butylmercapto)-ryanodine	>1200[j]				
117	9-epiryanodine	41[a]		13[k]		
118	8,9-didehydro-10-oxoryanodine	29.17[h]				
119 + 120	9,21-epoxy-ryanodine	140[a]	60[bm]		30[b]	
120	9β,21β-epoxy-ryanodine	29.17[k]				56.8[c]
121 A	9,21-dihydroxyryanodine	4900[a]		36[k]		
122	21-azido-9α-hydroxyryanodine		52[bm]	78[k]	25[b]	56.3[c]
124	21-*p*-nitro-benzamido-ryanodine		14.1[bm]	22[k]	19[b]	26.1[c]
125	21-bodipy-ryanodine			13[k]		
126	8,9-didehydroryanodine	13.46[h]	6.67[hn]			
131	8β-amino-9α-hydroxyryanodine		407[km]	760[k]	322[b]	
132	8α-amino-9β-hydroxyryanodine			3880[k]		
136	4,6-borate-dehydroryanodine	3.5[l]	4.19[ln]			
137	4,6-methylboronate-dehydroryanodine	15.91[l]	3.75[ln]			
138	4,6-phenylboronate-dehydroryanodine	7.45[l]				
139	4,6-cyclohexylboronate-dehydroryanodine	4.93[l]				
140	4,6-(3-thienyl)boronate-dehydryanodine	3.88[l]	2.61[ln]			
142	10,12-methylphosphonate-dehydroryanodine	83.33[l]				
144	10,12-N-methylphosphoramidate	77.78[l]		<51[l]		
148	2-deoxy-2(13)-dehydroryanodine	26.92[l]	5.81[ln]			
149 α	2-deoxy-2,13α-epoxyryanodine	250[l]	225[ln]			
149 β	2-deoxy-2,13β-epoxyryanodine	49.30[l]	22.78[ln]			
150	13-hydroxyryanodine	175[l]	45[ln]			
151	10-hydroxyiminoryanodine	13.46[h]				
153	10-epiryanodine	3.6[h]	2.31[hn]			
154	10-epi-amino-ryanodine	5.14[h]				
155	10-epi-benzamido-ryanodine	36.84[h]				
156	10-epi-hydroxyamino-ryanodine	10.61[h]				
158	10-epi-methoxyamino-ryanodine	5.14[h]	5.0[hn]			
162	10-epi-(4-azidobenzoyl)hydrazido-ryanodine	5.22[h]				
163	21-nor-9-oxoryanodine	35.0[h]	27.69[hn]			
164	21-nor-9$_{ax}$-hydroxyryanodine	564.5[h]	66.66[hn]			
167	21-nor-10-deoxyryanodine	53.85[h]				
168	10-deoxydehydroryanodine	9.46[h]	4[hn]			
170	21-nor-10-deoxy-9$_{ax}$-hydroxyryanodine	1750[h]	429[hn]			
171	lactam	8.33[h]	24.32[hn]			

Skeletal muscle IC_{50}, and skeletal and cardiac muscle K_D values were measured in rabbit tissues. IC_{50} for skeletal muscle calculated using competitive displacement of ryanodine by the ryanoid being tested. IC_{50} for cardiac muscle represents the concentration of the ryanoid that produced a 50% increase in the calcium permeability of SR membranes measured using the method of Fleischer et al. (1985). Fractional cond., Fractional conductance is the ratio of the chord conductance at +60 mV before and after modification of the sheep cardiac RyR reconstituted into a planar lipid bilayer. IC_{50}, concentration that inhibits 50%.

[a] Waterhouse et al., 1987.
[b] Mitchell et al., 1996.
[c] Tinker et al., 1996.
[d] Jefferies et al., 1992b.
[e] Bidasee et al., 1995.
[f] Kahl et al., 1994.
[g] Gerzon et al., 1993.
[h] Jefferies et al., 1996b.
[i] Jefferies et al., 1993.
[j] Jefferies and Casida, 1994.
[k] Welch et al., 1994.
[l] Jefferies et al., 1996a.
[m] IC_{50} was measured using porcine cardiac muscle.
[n] IC_{50} was measured in canine cardiac muscle.
[o] Welch et al., 1997.
[p] Welch et al., 1996.

atives has made it possible to begin to establish the specific features of the ryanodine molecule that are important for binding to the RyRs and for altering the properties of the RyR channels. The application of molecular modeling to investigations of the structure-activity relationships of ryanodine and related com-

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pounds is in its initial stages. In this section, we will describe the basis of this approach and the general findings that have been obtained through its use to date.

In this work, we have used quantitative structure-activity relationship analyses, in particular, comparative molecular field analysis (CoMFA) (Cramer et al., 1988). As a brief introduction, in the CoMFA method, a regular, three-dimensional grid of points is constructed around each of a selected group of molecules (the basis set). The physicochemical properties of the molecules are then sampled at each of the grid points. In principle, any number and kinds of properties can be sampled. For example, these could include hydrophobicity, frontier electron density, or other properties that may be related to the biological activities of the molecules comprising the basis set. In practice, usually two properties are assessed: the Lennard-Jones potential (to model Van der Waals forces) and the electrostatic potential. Differences in the physicochemical properties existing at the grid points for compounds in the basis set are correlated to differences in one or more biological activities. Because the number of grid points sampled is much greater than the number of observable properties being assessed, the analysis is overdetermined, and sophisticated statistical techniques are used. Commonly, partial least squares analysis is combined with cross-validation and bootstrapping techniques to test the quality of the analysis and eliminate correlations that arise only by chance. CoMFA is a versatile technique. It can be used to make quantitative correlations between molecular features and biological properties, to make quantitative predictions about the activities of compounds, to guide the synthesis of new compounds, to test hypotheses concerning the importance of specific structural features, and to probe the structural properties required for high-affinity binding and for altering the function of the receptor protein. In our hands, CoMFA has met the most important criterion of a structure-activity analysis: it has been used successfully to make predictions about properties of molecules not included in the basis set and has been applied successfully to data obtained in other laboratories. This predictive success supports the use of this approach to obtain insights into the nature of the ryanodine-RyR interaction.

The first step in applying CoMFA to a basis set of ryanoids is the determination of the conformation corresponding to the global energy minima of each of the compounds. While this can be difficult for flexible molecules capable of assuming a large number of conformations, the situation for many of the ryanoids is quite favorable, as the rigid nature of the polycyclic ring system precludes most of the potential conformational flexibility. In addition, the exocyclic functional groups on the ryanodine (**1**) molecule have extremely limited rotation. Application of a dihedral driver algorithm indicates that the energy surface has a well-defined global minimum; therefore, assignment of a conformation to the ryanoids is, in general, straightforward. Only the bond between the carbonyl and the pyrrole group has any ambiguity, as the torsional energy has two minima with comparable values. Even here, the choice of the proper conformation proved relatively clear and was consistent with experimental data published by other laboratories (see Welch et al. (1994) for further discussion of this point).

TABLE 2
Dissociation constants of ryanodine analogs used for the initial basis set

Compound Number	Compound	Rabbit K_D nM	Chicken K_D nM
1	ryanodine	7	7
55	dehydroryanodine	7	6
65	ester A	110	130
61	ester B	240	240
62	ester C_1	210	300
71	ester C_2	2600	3600
66	ester D	51	63
63	ryanodyl nicotinate	1100	1400
2	ryanodol	12000	3600
57	9-hydroxyryanodine	110	110
4	anhydroryanodine	2900	3000
70	anhydroester A	5200	8100
117	9-epiryanodine	13	13
120	9β,21-epoxyryanodine	36	29
122	21-azido-9-hydroxyryanodine	78	87
124	21-*p*-nitrobenzamido-ryanodine	22	22
125	21-bodipy-ryanodine	13	13
132	8α-amino-9β-hydroxyryanodine	3800	3800
131	8β-amino-9α-hydroxyryanodine	760	760

Rabbit and avian skeletal muscle K_D calculated using competitive binding of ryanodine against modified ryanodine in microsomal membranes.

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C. Correlations Between Ryanoid Structure and High Affinity Binding to the RyR

CoMFA conducted with a basis set of the 19 ryanoids shown in table 2 revealed several correlations between structure and the ability to bind to the high affinity site on the vertebrate RyR (Welch et al., 1994). The correlations are divided between those arising from the physical bulk (Van der Waal's contacts) and those arising from electrostatic properties. Both appear to contribute about equally to the correlations between structure and binding. From the correlations, it appears that the major electrostatic interactions are localized in the hydroxyl regions of the ryanoids. The major steric interaction appears to be in the pyrrole regions of the ryanoids. Addition of bulky and/or ionic groups to the 9–21-position and 10-position of ryanodine have minimal effects on the measured dissociation constants (table 1; Welch et al., 1994). These correlations have led to the proposal that the ryanoids bind to the RyR with the pyrrole locus buried within the ryanodine binding site and the 9-position and 10-position extending outside of the binding site (fig. 24; Welch et al., 1996). Note that lack of steric hindrance does not preclude thermal motions leading to interactions of flexible substituents at these positions with amino acid residues at or near the binding site (e.g. Witcher et al., 1994). Some of these features are examined in more detail in the following paragraphs.

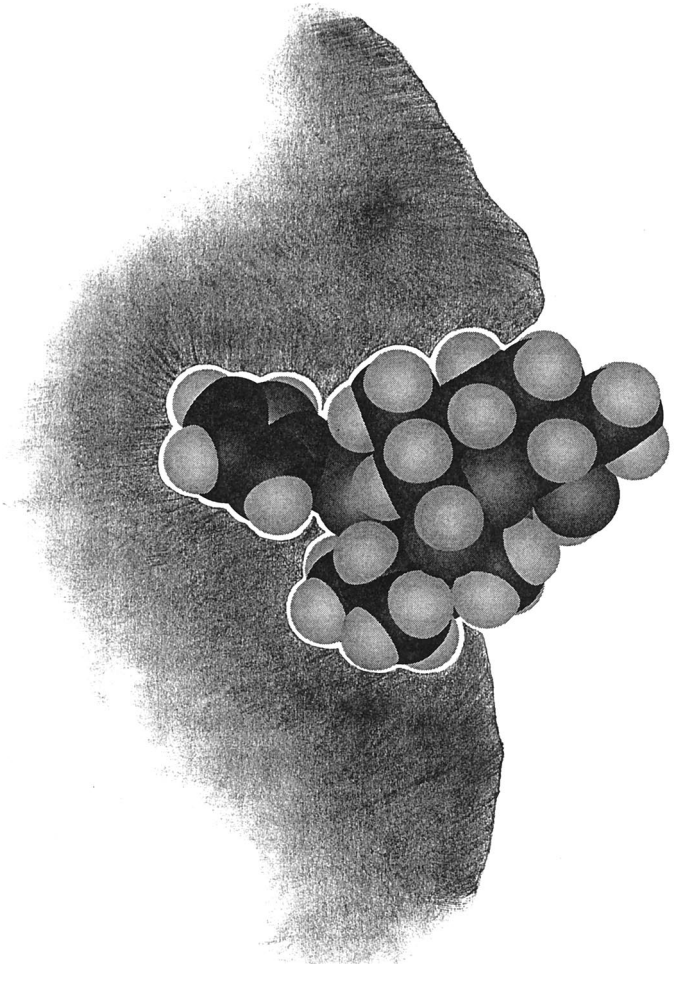

FIG. 24. A cartoon illustrating a possible orientation of the ryanodine molecule bound to the ryanodine receptor. The shaded area represents the protein; ryanodine is represented as a CPK (space filling) model with the pyrrole group at the far left.

*D. General Features of the Ryanodine (**1**) Molecule*

One application of CoMFA is to provide an explanation at the atomic level for the biological properties of a certain chemical. By multiplying the CoMFA field around ryanodine by the coefficients obtained from the partial least squares analysis of the basis set, one can identify molecular features of ryanodine responsible for its ability to bind with high affinity to the RyR. Such an analysis is shown in figure 25. The set of compounds used for the CoMFA is that used by Tinker et al. (1996) to measure fractional conductance. A ball-and-stick representation of ryanodine is shown in the middle panel. The wire frame on the left panel encloses the region of the ryanodine molecule where Van der Waal's interactions most strongly influence the binding of ryanodine. These areas are generally hydrophobic in character. The right-hand panel shows the area where electrostatic interactions account for most of the ryanodine-RyR binding interactions. This region is populated with multiple hydroxyl groups.

Can the regions of the ryanodine molecule associated with high affinity binding to the RyR be correlated with the physical properties of ryanodine? As indicated in the preceding sections of this review, ryanodine (**1**) has several interesting chemical properties. In seeking correlations between structure and biological functions, one is interested in physical properties such as lipophilicity, electrostatic potential, and hydrogen bonding potential. In preliminary investigations, we have visualized these properties with MOLCAD as implemented in SYBYL (Tripos Associates, St. Louis, MO). These properties are clearly segregated on the surface of the ryanoids. For example, ryanodine has a lipophilic face and a hydrophilic face (see Welch et al., 1994 for an illustration of this distribution). Although, overall, ryanodine is a neutral molecule, there are definite regions of positive and negative potential. As might be expected from the properties just described, hydrogen bond potential is located in well defined regions of the ryanodine molecule. We have compared these physical properties of ryanodine to the correlations between structure and binding (dissociation constants) obtained with CoMFA (figure 25) described in the preceding paragraph. We find that the steric factors that most influence the strength of binding of ryanodine to the RyRs are located in the lipophilic regions of ryanodine (and in regions of negligible hydrogen bonding potential). In contrast, there is no correlation between these steric factors and the electrostatic surface potential of ryanodine. The electrostatic factors affecting the strength of binding of ryanodine to the RyR

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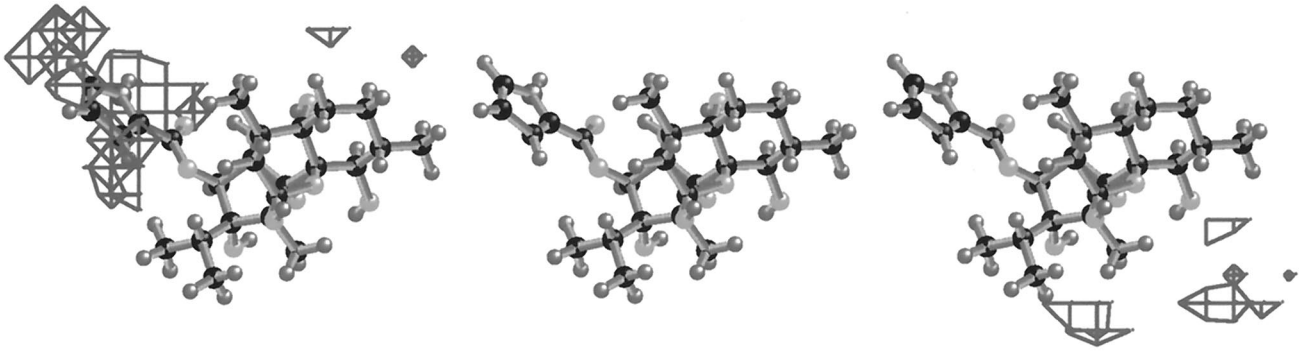

FIG. 25. Major loci responsible for high affinity ryanodine binding. A ball-and-stick representation of ryanodine (**1**) is shown in the middle panel. The pyrrole is located in the upper left, the isopropyl in the lower left, the 9-position (with the attached methyl group) is on the extreme right and the 10-position (with the attached hydroxyl) is immediately down and to the left. The wire-frame on the left panel encloses the region of the ryanodine (**1**) molecule where Van der Waal's interactions most strongly influence the binding of ryanodine. The right panel shows the area where electrostatic interactions account for most of the ryanodine-RyR binding interactions.

do not correlate to the lipophilic surface or to hydrogen bonding potential. The electrostatic factors affecting binding are generally concentrated in regions of negative surface potential, whereas there is little contribution of the positive surface potential to binding strength.

*E. The Pyrrole Group: A Primary Determinant of High Affinity Ryanodine (**1**) Binding*

Although ryanodine and ryanodol (**2**) have equivalent toxicities for certain insects, ryanodol is much less toxic to vertebrates than ryanodine (Jefferies et al., 1992a,b) and binds with an 1000 times lower affinity to vertebrate RyRs (Welch et al., 1994). This indicates that the pyrrole moiety is an important determinant for the binding of ryanodine to the vertebrate RyRs tested to date. Comparisons between ryanodine, ryanodol, and ryanodyl nicotinate (**63**, Ester G) establish this point further (Welch et al., 1994). Ryanodyl nicotinate differs from ryanodine by only the insertion of one carbon unit in the pyrrole ring, yet its binding to vertebrate RyRs is characterized by a 200-fold greater dissociation constant. No similarly sized alteration in any other exocyclic group tested has been found to have such profound effects on binding affinity. In the case of ryanodol, approximately 50% of the decrease in binding affinity can be assigned to a loss of hydrophobic interactions. The other factors are not yet known; however, preliminary modeling results indicate that an interaction involving the carbonyl oxygen of the pyrrole carboxylate group is important. This interaction may involve the formation of a hydrogen bond within the binding site of the RyR.

How important is the pyrrole group in the molecular recognition between ligand and receptor? To test whether the pyrrole carbonyl group is a dominant factor, we have assessed the consequences of moving this group to other regions of the molecule (Welch et al., 1996, 1997). For example, if the pyrrole group is a predominant determinant of high affinity binding, relocation of this group may have relatively minor effects on binding affinity. It was found experimentally that relocation of the pyrrole carbonyl group from the 3-position (ryanodine, **1**) to the 10-position (10-pyrrolecarbonyl ryanodol or 10-ryanodine (**44**)) or inversion of its configuration at the 3-position (3-epiryanodine, **50**) did not drastically increase the dissociation constant observed for binding to the vertebrate RyRs. The dissociation constants obtained for these isomers are less than those of either ryanodol (**2**) or ryanodyl nicotinate (**63**). Although it might be argued that favorable interactions between the RyR and the pyrrole-carbonyl group at the 10-position partially compensate for the loss of the pyrrole at the 3-position, recall that substitutions at the 10-position of ryanodine have relatively small effects on binding affinity (Bowling et al., 1994; Gerzon et al., 1993; Humerickhouse et al., 1994; Welch et al., 1994). Therefore, such an explanation seems unlikely. It appears that, despite the alterations in position of the pyrrole carbonyl group, ryanodine analogs containing this group are still able to achieve the favorable pyrrole-RyR contacts required for high affinity binding. In these cases, the pyrrole-carbonyl interactions with the RyR binding subsite are presumably sufficiently strong to cause the ryanodine isomer to bind in an alternate orientation that permits the pyrrole carbonyl to maintain its interaction with the binding subsite. The other ryanoid-RyR interactions would be sufficiently compliant as to allow the reorientation of the ligand within the binding site. Thus, maximal binding of a ryanoid to the high-affinity site appears to require the pyrrole carbonyl group. Replacement of the pyrrole carbonyl at the 3-position, the 3-epi position, or the 10-positions results in a loss in binding energy (Mitchell et al., unpublished observations). Substitutions that have been tested include small aliphatic groups, bulky groups, flexible and rigid groups, and charged substituents. It should be noted that the pyrrole group alone is not sufficient for binding to the high affinity ryanodine binding site, as pyrrole-1-carboxylic acid does not affect either [^{3}H]ryanodine binding or the Ca^{2+} permeability of SR membranes (Lattanzio and Sutko, unpublished observations).

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Recall that it has not as yet been possible to synthesize (+) ryanodine from (+) ryanodol (**2**); thus, the finding that the 10-ryanodine (**44**) and 3-epiryanodine (**50**) isomers bind with high affinity is particularly important to our modeling efforts. The synthetic strategies used to prepare these compounds provide a way to explore specific features of the pyrrole locus that do not exist using natural compounds.

The ability of CoMFA to predict the dissociation constants of ryanoids not included in the original basis was applied to the 3-epi and 10-ryanodine isomers (Welch et al., 1996). Multiple orientations were tested, and the calculated and experimental dissociation constants were compared. The analysis was consistent with the hypothesis that the pyrrole is the premier determinant of binding affinity and can cause reorientation of the ligand in the RyR binding site. What does this hypothesis suggest about the nature of the ligand-receptor interaction? The molecular volume common to these compounds excludes the isopropyl group (2-position), as well as the 9-position and 10-position. Exclusion of the 9-position and 10-position is consistent with experimental observations that relatively small changes in binding affinity result when large modifications are made at these positions (Bowling et al., 1994; Gerzon et al., 1993; Humerickhouse et al., 1994; Welch et al., 1994, 1997). On the other hand, as discussed in the next paragraph, exclusion of the isopropyl group at the 2-position is surprising. All of the ryanodine isomers have a common polar area along the nitrogen edge of the pyrrole that extends along the bridging carbonyl. There is a common hydrophobic area along the hydrocarbon edge of the pyrrole and on either side of the isopropyl locus (see Welch et al., 1996 for more details). It is tempting to speculate that these common polar and hydrophobic areas represent important forces directing the binding of ryanoid to the RyR binding site.

F. The Isopropyl Group at the 2-Position

Initial studies using CoMFA indicated that the isopropyl locus is also a major determinant of high affinity ryanodine (**1**) binding (Welch et al., 1994). Changes in the conformation of the isopropyl group are responsible for a large portion of the loss in binding affinity observed for anhydro ester A (**70**). The hydroxylation of this group (at position 18) in 18-hydroxyryanodine (**58**) is associated with a reduction in the ability of this compound (10% of the ryanodine potency) to promote calcium release from isolated SR vesicles (Jefferies et al., 1992a,b). It should be noted that the relationship between the inability of **58** to increase the permeability of SR membranes and its ability to bind to the RyR remains to be established (but see the last two sentences of this paragraph). In terms of binding constants, the consequences of changes made at the isopropyl group are not as great as that produced by the insertion of a comparable amount of bulk into the pyrrole locus (e.g., ryanodyl nicotinate (**63**), which has 200-fold lower affinity than ryanodine, **1**). One possible reason for the difference in the changes produced by alterations at these two sites may be that perturbations induced by the addition of a hydroxyl group at position 18 are localized to the isopropyl group, whereas CoMFA indicates that the presence of a larger group at position 3 also perturbs the polycyclic ring backbone of the ryanodine molecule. The CoMFA model also predicts that 18-hydroxyryanodine has a dissociation constant that is 16-fold greater than that observed for ryanodine compared with the 18-fold increase we have determined experimentally (Mitchell et al., manuscript in preparation). Thus, the reported effects of ryanodine, ryanodyl nicotinate, and 18-hydroxyryanodine on the Ca^{2+} permeability of rabbit skeletal SR membranes may parallel the strength with which these compounds bind to the RyR.

As noted in the preceding section, the isopropyl group at the 2-position is excluded from the volume common to the three isomers: ryanodine, 10-ryanodine and 3-epiryanodine. This would suggest that this group is not important for high affinity binding. Thus, there is an apparent contradiction between the data obtained for these three compounds and the predictions of the original CoMFA (Welch et al., 1994) as to the importance attributed to the isopropyl group for ryanoid binding. It should be noted that even when the three ryanodine isomers are included in the CoMFA basis set, the CoMFA continues to yield a strong correlation between binding and changes at the 2-position (Welch et al., unpublished data). All of the pyrrole-containing ryanodine analogs investigated have a common hydrophobic area along the hydrocarbon edge of the pyrrole and on either side of the isopropyl locus. Thus, the isopropyl group may be acting to provide an energetically important hydrophobic contact (see Welch et al., 1996 for additional details). The favorable hydrophobic interaction may be disrupted by steric hindrances when structural modification results in conformational alterations at the 2-position (e.g., anhydroryanodine (**4**), Welch et al., 1994 and see next section). In any case, the role of the 2-position in high affinity binding remains to be resolved.

G. Polycyclic Ring System

Our studies indicate that perturbations of the conformation of the polycyclic backbone induced by changes elsewhere in the ryanodine molecule may be a major cause of decreases in binding affinity (Welch et al., 1994). For example, CoMFA suggested that a significant fraction of the loss of binding observed for ryanodyl nicotinate (**63**) can be attributed to such changes. The importance of ring structure interactions is also evident in the case of anhydroryanodine (**4**), where the hemiacetal is replaced with a lactone structure. The structural changes in anhydroryanodine (**4**) also alter the conformation of the isopropyl group due to the presence of a double bond between atoms 1 and 2, and this also may

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contribute to the decrease in binding affinity of this compound. Similar statements can be made for the ester C2 (**71**), which has structural features and a binding affinity similar to those of anhydroryanodine (**4**).

The preceding results indicate that changes in the polycyclic ring structure influence interactions with the RyR that involve distant regions of the ryanodine (**1**) molecule. This observation appears to be inconsistent with a conclusion that was forthcoming from comparisons of the binding properties of ryanodine, 10-ryanodine (**44**), and 3-epiryanodine (**50**), namely, that the ryanodine binding site can accommodate the fused ring system in a variety of orientations (see beginning of section V.F.). The configurational aspects of the polycyclic ring structure in ryanodine responsible for the binding and efficacy of this compound as an effector of RyR channel properties remain to be established (see section V.J.). Based on present information, it appears that the fused ring system plays a passive, permissive role. It provides the necessary bulk to fill the ryanodine binding site and make nonspecific, but energetically important, hydrophobic and polar contacts. Alterations that leave gaps between ligand and receptor, or that cause configurational alterations near the critical pyrrole carbonyl locus, cause large losses in binding energy.

H. The 2-Hydroxyl Group

Alterations in the structural features can be context-dependent. The 2-hydroxyl group is relatively small and has relatively little effect on the conformation of the remainder of the ryanoid structure. If all ryanoids bind to the receptor in the same orientation, one could reasonably postulate that the contribution of the 2-hydroxyl-receptor interactions would remain constant. We have examined the effect of removal of the 2-hydroxyl group from different ryanoids. This group appears not to contribute to the binding of ryanodol (**2**). However, removal of this hydroxyl (see **53**) causes a five-fold increase in the dissociation constant of 3-epiryanodine (**50**). On the other hand, removal of this hydroxyl (see **54**) causes a two-fold decrease in the dissociation of 3-epiryanodol nicotinate (see **49C**) (Lindeken et al., unpublished results). These results are consistent with our conjecture that epimerization of the 3-position of ryanodine results in a major reorientation of the polycyclic ring system in the binding site (Welch et al., 1996). The reorientation is driven by the necessity for the pyrrole of both epimers to bind in the same orientation in the RyR binding site. Therefore, the 2-hydroxyl groups of 3-epiryanodol nicotinate and 3-epiryanodine appear to have different interactions in the ligand-receptor complex. In one case, the 2-hydroxyl group is positioned to make a favorable interaction; in the other, the interaction is not favorable.

I. Correlations Between Ryanoid Binding and Changes Induced in the Fractional Conductance of the RyR Channel

The CoMFA relating structural components of the ryanoids to the strength of binding have improved understanding of the nature of the interaction between ryanodine (**1**) and its high affinity binding site. All of the ryanoids tested to date affect the channel properties of the RyR. At low concentrations, these compounds cause the channel to exhibit partial conductances, and, at higher concentrations, most ryanoids also inhibit the channel (Mitchell et al., 1996). Tinker et al. (1996) found that changes in ryanodine structure resulted in large changes in the conductance properties of the sheep cardiac RyR (RyR2) channel. The results obtained indicate that different ryanoids cause RyRs to exhibit different fractional conductances. Furthermore, the lifetimes of the subconductance states varied with ryanoid structure. Therefore, the application of the computational techniques to the channel properties of the ryanoid-RyR complex was a logical extension of the previous work on binding. A CoMFA of the relationship between the structures of these compounds and their ability either to bind to the RyR or to modify RyR channel behavior revealed interesting contrasts (Welch et al., 1997). Whereas neither electrostatic nor steric alterations at the 9-position and 10-position had much effect on the binding constants, these alterations produced large decreases in the fractional conductance of the ryanoid-modified channel in electrophysiological measurements of channel properties. CoMFAs of binding and fractional conductance were performed on the same basis set of compounds. As noted previously for the larger basis set, steric and electrostatic factors contributed about equally to the correlation between structure and binding. In contrast, the CoMFA of fractional conductance indicated a three-fold stronger correlation between electrostatic factors and fractional conductance than between steric factors and fractional conductance. It is important to note that, whereas structure is correlated to both binding and fractional conductance, there is no correlation between dissociation constant and the fractional conductance of the ryanoid-modified RyR. In addition, the CoMFA of fractional conductance predicts the same relative binding orientations of the three ryanodine isomers (**1**, **44**, **50**) as does the CoMFA of binding affinity. The latter provides additional support for multiple binding orientations of ryanodine analogs in the receptor.

Figure 26 provides an atomic explanation of the properties of ryanodine (**1**) that lead to a reduced fractional conductance (the unmodified RyR is assigned a fractional conductance of 1). This figure is analogous to figure 25, which was obtained using the same basis set, and ryanodine is presented in the same orientation in both figures. The left-hand panel shows the principal region where steric factors modulate fractional conduc-

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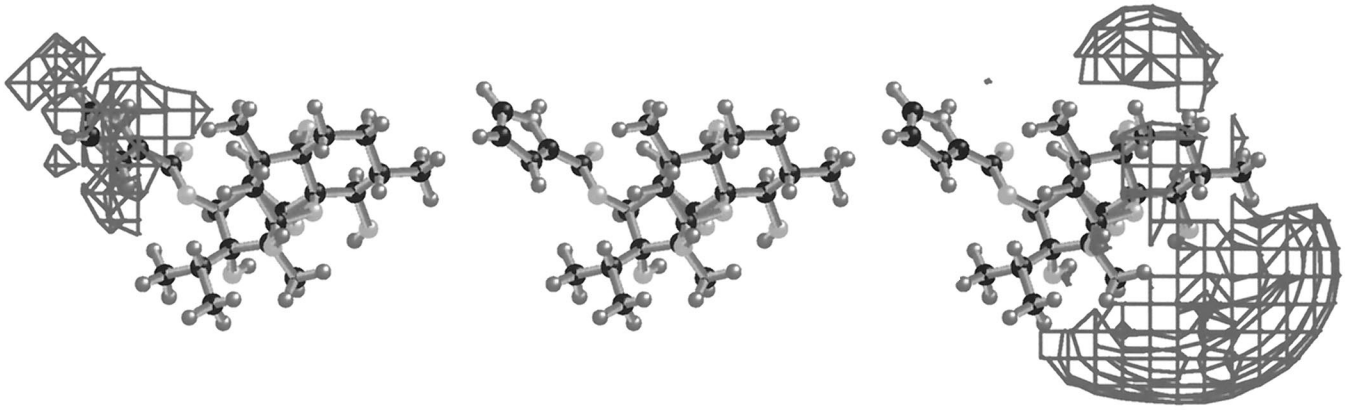

FIG. 26. Major loci responsible for decreased RyR conductance. Ryanodine (**1**) is represented as described in the legend for figure 25. See the text for a description of the meaning of the wire frames.

tance. Steric bulk at the pyrrole group favors smaller fractional conductances (closer to 0). The major electrostatic factors modulating fractional conductance are shown in the right-hand panel. These factors favor small fractional conductances. Note that the volume enclosed by the electrostatic factors is larger than the steric factors, reflecting the greater importance of the former (see paragraph above). The fractional conductance observed for ryanodine is the result of these two effects. The pyrrole contribution to fractional conductance is only slightly affected by modifications at the 9-position and 10-position. Therefore, the observed fractional conductances are primarily determined by the nature of the substituents at the 9-position and 10-position. It appears that one end (the 3-position) of the molecule primarily determines binding affinity, while the opposite end (9-position and 10-position) primarily controls channel behavior. If these correlations hold upon further testing, they suggest that it may be possible to modify one end of the ryanodine receptor to alter its binding properties and another region of the molecule to change its effects on the properties of the RyR channel.

A qualitative examination of correlations between the regions of ryanodine that contribute most to binding and the physicochemical surface properties of ryanodine was described earlier. The same kind of analysis was applied to the regions of ryanodine that contribute most to the observed fractional conductance and the surface properties of ryanodine. There were no obvious correlations between the regions making the majority of the electrostatic contributions to the fractional conductance of the ryanodine-RyR complex and the lipophilicity, electrostatic potential, or hydrogen-bonding potential. In contrast, the areas making the majority of the steric contributions to the fractional conductance were associated with areas of low hydrogen bonding potential and low electrostatic potential. There was no obvious correlation between the steric contributions and surface lipophilicity.

The partial conductances produced by the ryanoids could be due to allosteric effects that stabilize different RyR conformations, or they could result from direct interactions between the ryanoid and the permeant ion within the pore of the channel. The results of analyses of the magnitude of the steric and electrostatic correlations with fractional conductance conducted to date are not compatible with a direct interaction between the bound ryanoid and the permeant ion (Welch et al., 1997). The data we have obtained are more consistent with a model whereby the ryanoids modulate channel conductance through an allosteric mechanism.

J. Future Issues

The molecular modeling efforts conducted to date have contributed initial insights into the nature of the ryanodine-RyR interaction; however, many issues require additional attention. For example, the minimally effective structure capable of high affinity binding to the RyRs remains to be defined. In addition, the goal of ryanodine analogs specific for RyR isoforms has not yet been fulfilled. In general, the affinity of the ryanoids for RyR2 (found in cardiac muscle) is two-fold to three-fold higher than for the predominant RyR found in skeletal muscle, RyR1. The ratio of the binding affinities for the two RyR isoforms varies with ryanoid structure. Among the compounds analyzed, the ability to discriminate between these isoforms varies by as much as 10-fold. Preliminary CoMFA analyses indicate that RyR isoform-specific regions exist; however, it has not yet been possible to engineer ryanoids with sufficient isoform selectivity to make them useful experimentally. This remains an area of active interest.

Additional specific issues include clarification of the properties of the pyrrole carbonyl group that make this an important locus of ryanoid-RyR interaction. To this end, we have begun a series of variations on this pendant group. These include replacing the pyrrole with other ring systems that vary in dipole moment, electrostatic charge, and volume as well as with a series of flexible groups. The analyses of these analogs currently is in progress.

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Earlier in this article, CoMFA of the dissociation constants of 3-pyrrole carbonyl bearing ryanoids was compared with that obtained for the three ryanodine isomers (ryanodine, 3-epiryanodine, and 10-ryanodine). Superficially, the results appear contradictory. The initial CoMFA (Welch et al., 1994) indicated that the configuration of the polycyclic ring system was correlated with high affinity binding, thus suggesting specific fused ring-RyR interactions. The CoMFA of the dissociation constants and fractional conductances of the three ryanodine structural isomers strongly suggest that the polycyclic ring system can bind to the RyR in multiple orientations. These results suggest compliant interactions between the fused ring system and the RyR. To resolve the contradiction, we suggested that the polycyclic ring system provided a general framework for energetically important interactions. It was proposed that hydrophobic interactions were required in this region. Any hydrophobic group would suffice as long as there were no steric hindrances between receptor and ligand (Welch et al., 1996). However, the role of the isopropyl group, or any group adjacent to the 3-pyrrole carbonyl group, requires a more careful delineation.

As mentioned above, the physicochemical role of the polycyclic ring system requires clarification. The basic question relates to the specificity of the interaction with the receptor. Does the polycyclic ring system make multiple, specific contacts with the RyR binding site, or are the interactions general in nature? For example, do the hydroxyl groups of the fused ring system make essential and specific contacts, or are the required contacts permissive requiring only that they be polar and/or hydrophobic in nature. Data obtained to date support the latter possibility, but more rigorous analyses are required.

Previously, we described a qualitative comparison between the surface properties (lipophilicity, electrostatic potential, hydrogen bonding) of ryanodine and the molecular loci correlated with binding and fractional conductance. This analysis can be extended to a quantitative comparison at the atomic level of several ryanodine analogs in order to develop a mechanistic explanation of the interaction of ryanoid and RyR. A mechanistic understanding can abet mapping of essential ligand-receptor interactions and assist molecular design. Programs such as HINT (Kellogg et al., 1991) produce a three-dimensional matrix of hydrophobic and polar indices based on the Hansch relationships. These can be evaluated independently or as part of a larger CoMFA analysis. Hydrogen bonding potential can be evaluated through the use of alternate probe atoms in CoMFA. Such analyses do not necessarily improve the cross-validated correlation coefficients but are helpful in understanding the mechanisms underlying the CoMFA correlations. For example, these analyses will help to resolve the issues described earlier concerning the interactions between the RyR and the polycyclic ring system and the isopropyl group (2-position).

Estimations of the differences in solvation energies of the various ryanodine analogs are required. Interactions with solvent can be major factors determining the free energy of binding as measured from binding isotherms. Solvation factors are included implicitly in CoMFA. In fact, CoMFA has been able to predict successfully the acid dissociation constants of series of homologous compounds (Kim and Martin, 1991). Because crystallographic models of the RyR-ryanodine binding site do not exist, solvation energies cannot be included in a full thermodynamic cycle of binding. However, the explicit effects of structural changes on the ligand solvation free energy would provide an important parameter to be included in the structure analysis.

The seemingly anomalous behavior of some ryanoids may benefit from knowledge of solvation free energy. Although the dissociation constants of 9α-hydroxy-10-epiryanodine (**62**, Ester C1) and of other analogs containing modifications at the 10-position and 9–21-position are predicted well using our original basis set (Welch et al., 1994), the weak binding (Jefferies et al., 1992b; Welch et al., 1994) by the ester C1 (2 to 3% of that by ryanodine) is not readily explained. There are only minor differences in the dipole magnitudes and directions computed for these two compounds. Moreover, this is confounded by the observations made with other ryanoids that large structural modifications at the 10_{eq}-, and the 21-positions of the molecule have much smaller effects on binding (Bowling et al., 1994; Gerzon et al., 1993; Humerickhouse et al., 1994; Welch et al., 1994). One explanation is that changes in polarity at the 21-position and 10-position proximal to the ring structure produce significant changes in binding. In any case, these positions are likely to be directed outward from the binding pocket, as very large groups can be tolerated at both the 10-position (e.g., CBZ-alanyl derivative (**80**) and 21- (e.g., BODIPY (**125**)) position. Additional information is required for a good mechanistic explanation of the seemingly contradictory effects changes at these positions have on binding affinities.

Thus far, we have restricted our computational analyses to the high affinity ryanodine binding site, because this site has been reasonably well characterized. Simple, hyperbolic binding isotherms are routinely observed. In contrast, binding to the low affinity site is not clear-cut. Alternate mechanisms have been proposed: low affinity binding may arise from identical, interacting sites or from nonidentical, noninteracting sites. When the thermodynamic nature of the low-affinity site is defined, computational studies can proceed.

There is considerable variation in the fractional conductance of the ryanoid-modified RyR (Tinker et al., 1996). Strong structural correlations have been delineated in an initial panel of ryanodine analogs. The mechanism of the channel modulation remains to be eluci-

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dated. The two extreme (but not mutually exclusive) mechanisms are direct interaction between RyR-bound ryanoid and the permeant ion and ligand-associated conformational changes in the RyR. Quantitative structure-activity relationship analysis should be able to provide important evidence regarding the mechanisms by which the properties of the RyR channels are modified. Particular issues include the importance of the 9-position, 21-position, and 10-position in the ryanodine molecule, and how modifications in the molecule lead to alterations in channel properties, such as fractional conductance, open probability, mean lifetime of the closed and open states, and in the reversibility of the ligand-modified state of the RyR.

As was described in a previous section, we have found interesting correlations between ryanoid structure and the calcium flux through SR vesicles. The concentration of ryanoid required to cause half-maximal efflux through the RyR (EC_{50}) is essentially identical with the measured dissociation constants in all cases tested thus far, whereas the concentration of ryanoid required to half-close the channel (EC_{50}) varied from 5 to 1000 times the dissociation constant of the high affinity binding site (Mitchell et al., unpublished observations; Mitchell et al., 1996). These findings have important implications for the nature of the so-called low-affinity binding site. CoMFA of these observations are complimentary to the single-channel experiments and are currently underway.

K. Summary

Correlations between binding and structure are more advanced than correlations between calcium channel function and structure. Both have shown strong correlations between structure and biological activity. The results to date have led to a model of ryanodine-RyR interaction (fig. 24). The principal determinant of ryanodine binding is the pyrrole carbonyl locus. This group fits into a highly specific subsite within the ryanoid binding site on the RyR. The 9-position and 10-position are protruding from the binding site and are free to form interactions leading to modulation of RyR channel functions. The fused ring system plays a less specific role. It provides the necessary bulk to fill the ryanodine binding site and makes hydrophobic and polar contacts with the receptor. The binding site is sufficiently flexible to accommodate multiple configurations as long as deviations from the ryanodine shape are not too great.

VI. Nonryanoid Effectors of RyR Channel Function

RyR channel function is affected by a large number of chemically diverse compounds that are not structurally related to ryanodine. The majority of these compounds have been noted and discussed in several recent reviews (Coronado, 1994; Ogawa, 1994; Meissner, 1994). Therefore, as noted in the Introduction (section I), we have made ryanodine and related compounds the focus of this article and the readers are referred to these earlier reviews for this information. In this section, we note compounds that have either been described since the preceding reviews appeared or for which there has arisen some new information or controversy concerning the nature of their actions on the RyRs.

A. Proteins

1. FK 506 binding proteins. As discussed previously, the immunophilin FK506 binding proteins (FKBPs) have been shown to bind tightly to the RyRs in an association that influences RyR channel properties (Jayaraman et al., 1992; Brilliantes et al., 1994; Timerman et al., 1993, 1994, 1995). The nature of the interaction between the FKBP and the RyR is not known. FKBP exhibit *cis/trans*-peptidyl-prolyl isomerase activity; therefore, the possibility exists that FKBP could induce changes in RyR conformation via this activity. This does not appear to be the case though, as Timerman et al. (1995) exchanged a mutant FKBP that lacked measurable isomerase activity for the wild type protein normally complexed with the RyR and found no change in RyR properties. The association of FKBP with the RyR has been shown to result in a decreased probability of RyR channel opening and to decrease the occurrence of openings to fractional conductance levels. These effects lead to a decrease in SR membrane Ca^{2+} permeability. More recent data indicate that FKBP binding can also make conduction by the RyR channel asymmetrical (Chen et al., 1994; Ma et al., 1995). In the presence of FKBP, current flow from the SR lumenal to the cytoplasmic side of the channel (as would occur during SR Ca^{2+} release) was unaffected, but that from the cytoplasmic to the lumenal side of the channel is reduced.

Members of a family of macrocyclic compounds isolated from the sponge *Ianthella basta*, termed bastadins, have been shown to increase the [^{3}H]ryanodine binding capacity and the Ca^{2+} permeability of SR membranes (Mack et al., 1994). On a single RyR channel level, a marked prolongation of channel open time was observed. The effects of these compounds on RyR channel properties were antagonized by the immunosuppressant drug, FK506, which causes FKBP to dissociate from the RyR. The bastadins by themselves did not cause FKBP to dissociate, but enhanced this effect of FK506. It is suggested that the bastadins produce their effects by binding to the FKBP and altering the interaction between the latter protein and the RyR (Mack et al., 1994). Thus, these compounds may provide useful tools with which to define the nature of the FKBP-RyR interactions.

2. Calmodulin. The actions of calmodulin on the RyR channel are complex. At low concentrations of Ca^{2+}, this agent activates the channel, whereas at high Ca^{2+} concentrations, the channel is inhibited (Tripathy et al., 1995; Ikemoto, 1995). Thus, calmodulin may serve as a switch controlling RyR-mediated Ca^{2+} release in a need-

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dependent manner. Recent investigations have found that calmodulin antagonists, such as trifluoperazine, chlorpromazine, W-7, mastoparan, and a calmodulin-binding peptide derived from Ca^{2+}/calmodulin-dependent protein kinase II, do not prevent the biphasic effects of calmodulin on RyR channel activity (Ikemoto, 1996). Thus, calmodulin may interact with the RyR or a RyR-associated protein in a different manner than it does with other proteins, where its interactions are sensitive to these inhibitors. In addition, the above-named calmodulin inhibitors were found to activate the Ca^{2+}-induced release of SR Ca^{2+} in skinned rabbit skeletal muscle fibers, an effect that appears to be independent of the involvement of calmodulin (Ikemoto, 1996). These data emphasize further the complexities of the actions of calmodulin on the RyR and of the pharmacology of the RyR itself.

3. Triadin. Triadin, an integral SR membrane protein, has an as-yet-unresolved role in skeletal muscle function. It has been proposed to interact with, and to transmit an activation signal between, the DHPR and the RyR (Kim et al., 1990; Brandt et al., 1990; Caswell et al., 1991). Alternatively, it has been proposed to interact with both the RyR and the SR lumenal Ca^{2+} binding protein, calsequestrin, and perhaps to coordinate SR Ca^{2+} storage and release activities (Guo et al., 1994, 1995). Recent data suggest that the redox state of sulfhydryl groups in both the RyR and triadin serve as determinants of the interactions between these proteins (Liu et al., 1994; Liu and Pessah, 1994). Changes in oxidation state have been shown in numerous studies to influence RyR-mediated SR Ca^{2+} release, and alterations in triadin-RyR interactions may provide at least a partial mechanism for these effects.

4. Sorcin. Sorcin, a 22-kDa Ca^{2+} binding protein, whose expression is up-regulated in cultured cells during the acquisition of multidrug resistance, has been shown to be localized to the SR in cardiac muscle, where it appears to be associated with the RyR (Meyers et al., 1995). The nature of the interactions between these proteins and the consequences this has on RyR function remain to be elucidated.

B. Peptide Toxins

1. Myotoxin a. Myotoxin a, a polypeptide isolated from the venom of the prairie rattlesnake, increases the Ca^{2+} permeability skeletal muscle SR membranes and appears to be capable of activating the RyR channel (Yudkowsky et al., 1994; Furukawa et al., 1994).

2. Helothermine. Helothermine, a 25.5-kDa polypeptide from the venom of the Mexican beaded lizard, has been shown to block both skeletal and cardiac RyR channels and has been cloned and sequenced (Morrissette et al., 1995).

3. Ryanotoxin. Ryanotoxin, a peptide isolated from scorpion venom, has been shown to have effects on RyR channel activity that are similar to some of those produced by ryanodine (Morrissette et al., 1996). In particular, the ryanotoxin peptide increased the release of Ca^{2+} from SR membrane vesicles and induced a long-lived subconductance state in isolated RyR channels. Ryanotoxin stimulated [^{3}H]ryanodine binding in a concentration-dependent and a reversible manner; thus, it is unlikely that the peptide interacts directly with the ryanodine binding site.

4. Imperatoxin A. Imperatoxin A, a peptide isolated from the venom of a scorpion, has been shown to have RyR isoform-specific effects in that it can selectively activate the RyR1 channel, while not effecting the Ca^{2+} permeability of microsomal membranes isolated from tissues expressing other RyR isoforms (Arevalo et al., 1996; El-Hayek et al., 1995b). The properties of this toxin indicate the potential for isolating and/or designing isoform-specific peptide effectors of the RyR channels. A potential limitation of this class of agents is that they are generally nonpermeant to cell membranes and, therefore, ineffective when applied extracellularly.

C. Cyclic Adenosine Diphosphate Ribose

The seminal studies by Lee and colleagues (Clapper et al., 1987; Dargie et al., 1990; Lee, 1996) and, subsequently, those by many other laboratories have demonstrated that cyclic adenosine diphosphate ribose (cADPR) is an intracellular messenger capable of releasing Ca^{2+} from cellular stores in a variety of tissues. The results of several studies using different tissues suggest that cADPR produces this effect by activating a RyR-mediated Ca^{2+} release process. This is the case in cardiac muscle, where it has been proposed to be an endogenous regulator of SR Ca^{2+} release (Meszaros et al., 1993) and shown to increase the channel activity of the cardiac RyR isoform, RyR2 (Sitsapesan et al., 1994). The physiological importance of the actions of this agent has been questioned because of a lack of observable effects of cADPR on RyR function in cardiac SR membranes in vitro (Fruen et al., 1994), or when this agent was introduced into isolated cardiac myocytes (Guo et al., 1996). Also, it has been questioned whether actions of cADPR observed in vitro would be observed in the intact cell, if this agent binds to the nucleotide binding site on the RyR, a site that may be saturated with ATP under normal in vivo conditions (Sitsapesan et al., 1994, 1995; Sitsapesan and Williams, 1995). In view of the preceding observations, the role of cADPR as an intracellular messenger in cardiac muscle remains controversial.

D. Local Anesthetics

RyR channel properties have been shown to be affected by a variety of local anesthetics (Coronado et al., 1994). Recently, both propranolol (Zchut et al., 1996) and cocaine (Tsushima et al., 1996), compounds which possess local anesthetic properties, have been reported to influence also the activity state of RyR channels.

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E. Polyamines

Millimolar concentrations of polyamines that occur endogenously in many cells, such as spermine, spermidine, and putrescine, have been shown recently to cause a rectification of rabbit cardiac RyR channels (Uehara et al., 1996). These agents were found to act from both sides of the channel, and the efficacy of the block they produced was dependent on the direction of current flow and on the magnitude of the potential imposed across the bilayer; the efficacy was also inversely related to the ion selectivity of the channel. It is suggested that polyamines act as permeable cationic blockers of the RyR channel. The concentrations of polyamines reported to exist in muscles would permit them to serve as endogenous regulators of RyR channel activity via this effect.

F. Suramin

Suramin, a polysulfonated naphthylamine derivative of urea, has been used as an antiparasitic agent for many years and recently has been shown to inhibit reverse transcriptase in retroviruses. Suramin is also a competitive inhibitor of ATP at the P_{2x} purinergic receptors (Voogd et al., 1993). Interestingly, this agent can modify also the channel properties of the RyRs. Suramin increases the Ca^{2+} permeability of skeletal muscle terminal cisternae SR membranes (Emmick et al., 1994) and activates both the rabbit skeletal muscle RyR1 and sheep cardiac RyR2 isoform channels reconstituted into planar lipid bilayers (Sitsapesan and Williams, 1996). The increase in channel activity was primarily due to a large increase in the mean open time of the channel. A 20 to 25% increase in conductance also was observed. Interestingly, suramin was approximately 10 times more potent at effecting RyR2 channels. As noted by the authors, this difference in potency could be either due to a true difference in affinity with which suramin binds to the RyR1 and RyR2 isoforms or to differences in the intrinsic channel properties of these RyRs that are affected by suramin (Sitsapesan and Williams, 1996). In either case, suramin-related compounds may have the potential for producing RyR isoform-specific effects. Although, as noted above in this paragraph, suramin is an ATP antagonist, it does not appear to interact with the ATP site on the RyRs (Sitsapesan and Williams, 1996). Therefore, suramin and perhaps related compounds may provide a unique class of modifiers of the RyR channels.

G. Ortho-*Substituted Polychorinated Biphenyls*

Polychorinated biphenyls are a family of aromatic hydrocarbons used extensively in industry that has become a significant environmental contaminant. Recent studies indicate that *ortho*-substituted polychorinated biphenyls are capable of activating the release of calcium from skeletal and cardiac SR membranes in a ryanodine-sensitive and ruthenium red-sensitive manner. In addition, these compounds enhanced the binding of [^{3}H]ryanodine to membranes from both types of muscle (Wong and Pessah, 1996). Preliminary studies described by these authors suggest that the actions of these compounds may be similar to those of the bastadins noted above, in that they are dependent on the presence of FKBP-12.

VII. Footprints in the Sand

In concluding this review, we would like to note the contributions made by two workers to advances that have been made in this area.

The first are those made by Dr. Alan Fairhurst in collaboration with Dr. Donald Jenden and colleagues. Dr. Fairhurst made several seminal observations related to the action of ryanodine. Among these was the ability of ryanodine to increase the Ca^{2+} permeability of SR membranes (Fairhurst and Hasselbach, 1970; Fairhurst, 1974). One of the primary actions of ryanodine on the RyR channel should have been recognized much earlier than it was, based on these results. Dr. Fairhurst devised one of the two ways that have been used for incorporating tritium into the ryanodine molecule (Fairhurst, 1971). The availability of [^{3}H]ryanodine has permitted identification and purification of the RyRs, as well as the characterization of many functional aspects of these proteins. Finally, he was also among the first to demonstrate regional heterogeneity in the SR by showing that both heavy (ryanodine-sensitive) and light (ryanodine-insensitive) fractions of SR membranes could be obtained by differential centrifugation (Fairhurst, 1974).

The second series of contributions are ones made by Dr. Pierre Deslongchamps and coworkers to the isolation and identification of naturally occurring ryanoids, and to the elucidation of the chemical properties and the development of the strategies necessary for the synthesis of the ryanoid, ryanodol (Belanger et al., 1979; Deslongchamps et al., 1990). Although recognized for its creativity and ingeniousness in the field of organic synthesis, this work is now also being appreciated for the wealth of ryanoids and ryanoid intermediates that it has produced. The latter compounds have provided the basis for many of the ryanodine structure-function analyses that have been conducted to date.

It should be noted that the contributions made by both Drs. Fairhurst and Deslongchamps predate the re-emergence of ryanodine as an interesting experimental probe and have provided an important and solid basis for the current rapid advances that are being made concerning molecular, pharmacological, and chemical aspects of the actions of ryanodine and the focus of its effects, the RyRs.

VIII. Summary

The goal of this review has been to describe the current state of the pharmacology of ryanodine and related

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compounds relative to the vertebrate RyRs. Resolution of questions concerning the molecular properties of RyR channel function and the contributions made by the RyR isoforms to cellular signaling in a variety of tissues will require the production of new pharmacological agents directed against these proteins. Novel naturally occurring ryanodine congeners have been identified, and significant advances have been made in developing chemical approaches that permit the structure of ryanodine to be derivatized in selective ways. Moreover, several of these changes have yielded compounds that differ in their binding affinities and in their abilities to modify the properties of the RyR channels. These advances give substance to the possibility of designing the required pharmacological agents based on rational design changes of the structure ryanodine.

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