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AN EVALUATION OF METHYLENEDIOXYINDENE ANALOGUES IN CARDIAC MUSCLE SYSTEMS

The Ohio State University

Ph.D. 1982

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AN EVALUATION OF METHYLENEDIOXYINDENE ANALOGUES
IN CARDIAC MUSCLE SYSTEMS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Joseph John Lynch Jr., B.Sc.

* * * * *

The Ohio State University
1982

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Mammalian myocardial ultrastructure

The structure of working myocardial cells (the predominant cell type in cardiac muscle, specialized for contraction as opposed to Purkinje fibers or nodal cells which are specialized for conduction or pacemaker activity) has been extensively described (McNutt and Fawcett, 1974; Sommer and Johnson, 1979; Adams and Schwartz, 1980). The cells of the working myocardium (myofibers) appear microscopically as fibers, each enclosed in its own plasma membrane (sarcolemma). Myofibers bifurcate extensively, connecting with adjacent fibers end-to-end by means of tight junctions known as intercalated discs to form an intricate, three dimensional network. Myofiber sizes range between 50 to 100 micrometers in length and 10 to 20 micrometers in diameter. The predominant structural feature of cardiac myofibers are the myofibrils contained within, and filling the sarcolemma. Myofibrils appear as bundles of thin longitudinal elements which have a repeating pattern of light and dark transverse bands, giving the cardiac myofiber a striated appearance. Myofibril structure and the
contractile process will be discussed at length in a subsequent section. Other predominant structures within the myofiber include mitochondria, a centrally located nucleus, an extensive internal membrane system (sarcoplasmic reticulum) and tubular invaginations of the sarcolemma (transverse tubules, or T-tubules).

The sarcolemma of the cardiac myofiber is a basic cell unit membrane, having a bilayered structure of 7 to 9 nm thickness. On the exterior of the sarcolemma is the glycocalyx, which is 50 nm thick, and is composed of at least two layers (Bennett, 1963). The inner layer, known as the surface coat, is about 20 nm thick and is an extension of the sarcolemma. The more peripheral layer, known as the external lamina, is about 30 nm thick and extends from the surface coat (Langer, 1978). The glycocalyx, due to its chemical composition (anionic mucopolysaccharides, glycoproteins, sialic acid residues), is thought to represent an extracellular region with a high capacity for cation binding, particularly calcium, and thus may be a site of calcium binding and exchange across the sarcolemma, playing a role in cardiac muscle excitation-contraction coupling.

The sarcolemma of cardiac myofibers is vesiculated and indented at regular intervals. The indentations represent sites of tubular membrane invaginations of the sarcolemma (and of glycocalyx and extracellular space) into the
cell interior and comprise what is known as the transverse tubular system (T-tubules). In cardiac muscle, the T-tubules invaginate predominantly at the Z-line of sarcomeres, penetrating to the center of the myofiber and occasionally bifurcating to extend longitudinally between adjacent myofibrils (Sommer and Johnson, 1979).

Embedded within the sarcolemmal membranes of the cardiac myofiber are numerous macromolecules that have been identified to play important roles in the regulation of cardiac muscle contraction. These membrane-bound macromolecules include receptor sites for hormones, neurotransmitters and drugs (Jones et al., 1979; Rash and Ellisman, 1974; Schmalbruch, 1979), as well as membrane-bound enzymes such as Na\(^+\)/K\(^+\)-ATPase (Schwartz et al., 1975; Pitts et al., 1977), adenylate cyclase (Lefkowitz et al., 1976; Watanabe and Besch, 1975) and a Ca\(^{2+}\)-ATPase for extrusion of intracellular calcium ion out of the cell (Caroni and Carafoli, 1980; Trumble et al., 1980). Two other constituents of the cardiac myofiber sarcolemma, ion channels and a Na\(^+\)-Ca\(^{2+}\) countertransport site, will be dealt with in a later section.

A unique structural feature of the cardiac myofiber is the intercalated disc. The intercalated disc is a specialized structure which is thought to serve as a low resistance pathway between adjacent cells, thus facilitating electrical current flow during excitation. This
A structural feature gives the myocardium the characteristic of an electrical, as well as functional syncytium (Adams and Schwartz, 1980).

The mitochondrial content of cardiac myofibers is quite high, with this organelle occupying 55% of cell volume. (Sommer and Johnson, 1979). Mitochondria are located beneath the sarcolemma, as well as around and between the myofibrils. Usually associated with or close to mitochondria are lipid droplets and glycogen granules, both of which contribute to the energy requirements of cardiac muscle, which is primarily dependent upon oxidative phosphorylation by the mitochondria. The possible participation of mitochondria in the regulation of intracellular calcium during the excitation-contraction coupling process is discussed in a later section.

The intracellular membrane system, the sarcoplasmic reticulum (SR), of cardiac myofibers is discontinuous with the sarcolemma, but continuous with internal membranes such as the nuclear envelope (Sommer and Johnson, 1979). Myocardial SR, which is predominantly of the smooth type, consists of a plexiform arrangement of tubular elements which surround the myofibrils. Myocardial SR is connected at virtually all levels of the sarcomere, and therefore tends to have a somewhat random orientation with respect to the axis of the myofiber. The SR in cardiac muscle terminates in dilations (termed subsarcolemma cisternae)
which closely oppose the membrane of the T-tubules and the sarcolemma via junctional processes. The close structural association between extracellular space (including the T-tubules) and intracellular structures such as SR and myofibrils suggests a close functional association in the process of excitation-contraction coupling (Adams and Schwartz, 1980).

A major enzymatic constituent of cardiac SR membrane is a Ca$_2^+$-ATPase, which constitutes the enzymatic machinery of the SR calcium pump. The SR-Ca-ATPase has a high affinity for Ca$_2^+$ which allows for rapid rates of binding and subsequent Ca$_2^+$ transport across the membrane during relaxation (Adams and Schwartz, 1980). It is possible that reversal of the pump and/or changes in SR membrane permeability may occur when Ca$_2^+$ is released to the myoplasm to elicit contraction (Endo, 1977; Hasselbach, 1978). The participation of SR in the regulation of intracellular calcium during the excitation-contraction coupling process is discussed in a subsequent section.

Cardiac myofibrils and contraction

The most distinctive structural feature of cardiac cells is the regular alternating array of transverse bands within the cytoplasm, which are the myofibrils, the contractile substance of the cell. The myofibrils are arranged parallel to the axis of the muscle fiber and are
contained in repeating units called sarcomeres. The sarcomere is delineated by two successive narrow dark lines known as Z-lines. The distance between sarcomeres in resting cardiac muscle is on the order of 2 to 3 micrometers. Between any two successive Z-lines occurs one dark, centrally located band known as the A-band (anisotropic, or birefringent band). At either end of the A-band are the more lightly staining sections known as the I-bands (isotropic band), which are bisected by the Z-lines. Hence, the sarcomere, the repeating structural unit to which all morphological events of the contractile cycle are referred, defined as the segment between two successive Z-lines, contains one A-band and half of the two contiguous I-bands. Additionally, when cardiac muscle is relaxed, a pale zone called the H-band may be discerned traversing the center of the A-band. In its center is a narrow dark line, the M-line, located precisely in the middle of the A-band (Bloom and Fawcett, 1975).

The thin, parallel cross-striated myofibrils which are the contractile substance of cardiac cells have been found in recent years to be composed of smaller units known as myofilaments. These are of two kinds, differing in dimension and chemical composition. The cross banded pattern of the cardiac myofiber reflects the arrangement of these two sets of submicroscopic filaments. The thicker myosin filaments, 10 nm in diameter and 1.5 micrometers
long, are parallel and about 45 nm apart. The parallel arrays of myosin filaments are the principal constituent of the A-band and determine its length. The myosin filaments are slightly thicker in the middle, taper toward both ends, and are held in register by cross connections that are aligned at the midpoint of the A-band, giving rise to the transverse density known as the M-line. The thinner actin filaments, 5 nm in diameter, attach to, and extend, 1 micrometer in either direction from the Z-line, thus constituting the I-band. The actin filaments are not limited to the I-band, however, in that they extend into the adjacent A-bands, overlapping and interdigitating with the myosin filaments. The depth to which the ends of the actin filaments penetrate into the A-band varies with the degree of contraction. In the relaxed state, the thin actin filaments that extend into the A-band from opposite ends do not meet. The distance between their ends determines the width of the pale H-band, the zone within the A-band which is composed of only thick myosin filaments. Conversely, the I-band may be defined as that area surrounding the Z-line which is composed of only thin actin filaments (Bloom and Fawcett, 1975). The widths of the I-bands and H-bands are not constant, but rather vary according to the degree of actin (thin filament) and myosin (thick filament) overlap. When the myofiber is relaxed, there is minimal overlap, and the H-bands and
I-bands become wider. When the myofiber is contracted, however, both regions become more narrow. In either case, the width of the A-band remains constant, indicating that the myosin filaments (as well as the actin filaments) do not undergo a change in length during cardiac muscle contraction and relaxation, but rather that contraction and relaxation are the result of changes in the degree of overlap of actin and myosin filaments. This latter observation is the foundation for the sliding-filament theory of muscle contraction (Huxley and Niedergerke, 1954; Huxley and Hansen, 1954).

Stated briefly, the sliding-filament theory suggests that myofiber contraction is brought about when interdigitating thick myosin and thin actin filaments slide past each other. Shortening of the myofiber and concomitant tension development occurs as the result of cyclic reactions between myosin filament projections (visible with very high magnification electron microscopy) and active sites on the actin filaments. Each myosin projection initially attaches to an actin filament to form a cross-bridge. Then it moves or contracts, and then releases, being in a position to attach to another site immediately adjacent on the actin filament. Hence, the actin filaments are drawn into the myosin A-band, adjacent Z-lines are drawn closer to each other, and there is an overall shortening of the myofibril. The energy for this
physical process is provided by ATP and is released by the interaction of actin with myosin, which activates myosin-ATPase activity (Adams and Schwartz, 1980).

An elucidation of the composition of the myofilaments and their mode of interaction (Adelstein and Eisenberg, 1980) has provided a biochemical correlate for the sliding-filament theory for muscle contraction. Myosin, the thick filament constituent, is a hexameric protein (460,000 dalton) consisting of one pair of heavy chains (200,000 dalton) and two pairs of light chains (15,000 to 27,000 dalton) (Lowey, 1979). One pair of the light chains on each myosin molecule appears to be required for myosin-ATPase activity (this pair termed the essential light chains), while the second pair has been suggested to play a regulatory role in the actin-myosin interaction, a role which will be discussed in a later section. The myosin molecule has unique enzymatic and structural properties which are integral to its role in the contractile process. The myosin molecule is grossly assymetric in that the amino terminal portion of the myosin heavy chain is globular in shape, while the carboxyl terminal portion is fibrous. The fibrous end is almost completely alpha-helical and is responsible for the formation of the thick myosin filament, while the globular portion of the myosin molecule, which binds to actin and contains the site of ATPase
activity, constitutes the myosin projection which reacts with the actin filament (Lowey, 1979).

Actin (43,000 dalton), the major constituent of thin myofilaments, is a globular protein that polymerizes to form a double helical filament at physiological ionic strength. All of the actin in cardiac myofiber is found in a filamentous form. Besides its structural role in myofibril composition, actin is also capable of activating the extremely low rate at which myosin hydrolyzes ATP at physiological ionic strength in the presence of Mg$^{2+}$. A second protein found in the thin actin myofilament is tropomyosin. Tropomyosin from cardiac muscle has a molecular weight of 66,000 daltons and is composed of two alpha-helical subunits that coil about each other. Tropomyosin molecules lie end to end in the grooves created by the dual strands of actin in the thin myofilament. Tropomyosin, in cooperation with troponin (below) acts to inhibit myosin-actin interaction. The third component of the thin actin filament is the protein troponin, which is found at regular intervals (every seven actin monomers) along the thin actin filament in a particular spatial arrangement with tropomyosin. Troponin exists as a globular complex of three proteins: troponin I (24,000 dalton) which acts as an inhibitor of actin-myosin interaction; troponin T (35,000 dalton), which binds the troponin complex to
tropomyosin; and troponin C (18,000 dalton), which contains binding sites for Ca$^{2+}$ and Mg$^{2+}$. (Adelstein and Eisenberg, 1980).

Analysis of the interactions between the myofilament proteins described above have revealed three major characteristics of cardiac muscle contraction: 1) the occurrence of ATP hydrolysis and the subsequent liberation of energy during contraction; 2) the alteration of physicochemical properties that, associated with ATP hydrolysis, result in muscle shortening and the development of tension; and 3) the dependence on calcium ion in initiating and regulating contraction, primarily via an actin-based regulation of actin-myosin-ATP interaction (Weber and Murray, 1973; Van Winkle and Schwartz, 1976).

As set forth by the sliding-filament theory for muscle contraction, contraction consists of the cyclic attachment and detachment of the globular portion of the myosin molecule to the actin filament. The attachment is followed by a change in the angle of myosin-actin attachment, so that the thick and thin myofilaments slide past each other. The energy for the process is provided by ATP hydrolysis by the myosin-ATPase located on the globular portion of myosin. Purified myosin is known to hydrolyze ATP at a very slow rate in the absence of actin. The addition of actin to purified myosin, however, increases
the rate of ATP hydrolysis 100-200 fold. This activation of myosin ATPase occurs whether or not Ca$^{2+}$ is present. Regulation of the actin-myosin interaction (and actin-stimulated myosin-ATPase activity) is imposed on cardiac muscle by the presence of the troponin-tropomyosin complex on the actin thin filament (hence an actin-based regulation); the addition of these proteins to an actin-myosin mixture suppresses actin-activated myosin-ATPase activity at concentrations of Ca$^{2+}$ present in the relaxed state of myofibers ($<10^{-7}$M). A rise in Ca$^{2+}$ to $10^{-5}$M, which occurs in the myofiber during contraction, relieves this suppression (Weber and Murray, 1973; Perry, 1979). In the myofiber, it is thought that the rise in Ca$^{2+}$ concentration which accompanies contraction serves to make Ca$^{2+}$ available to binding sites on troponin C. Calcium binding to troponin C, in turn, inhibits or reverses binding of troponin I to actin. With Ca$^{2+}$ bound to troponin, the tropomyosin molecule undergoes a conformational change from one which blocks interaction between actin and myosin to one which promotes interaction. The formation of an active complex of actin and myosin is dependent upon ATP hydrolysis by myosin, which is activated by the actin-myosin interaction. Associated with release of phosphate-bound energy, there is a change in the angle of the actin-myosin attachment, which results in a positional shift of the actomyosin complex, drawing the thin filaments toward the center of the
sarcomere, which is, in essence, contraction. Relaxation, conversely, accompanies a reduction in the cystosolic Ca\(^{2+}\) concentration, which favors the dissociation of Ca\(^{2+}\) from troponin, which in turn results in a renewed hindrance of actin-myosin interaction. In summary, in cardiac muscle, there is a Ca\(^{2+}\)-independent actin-activated myosin-ATPase activity which is repressed by the binding of troponin-tropomyosin to actin and is derepressed by Ca\(^{2+}\)-binding to troponin (Weber and Murray, 1973; Perry, 1979).

**Extracellular calcium and cardiac contractility**

Given the major role played by Ca\(^{2+}\) in the actin-based regulation of actin-myosin-ATPase activity, it is not surprising that Ca\(^{2+}\) plays an essential role in the initiation and development of contraction in cardiac muscle. Early work established that, as opposed to skeletal muscle with which cardiac muscle shares several structural and functional features (Fleckenstein, 1977), cardiac contraction has an absolute dependence upon an extracellular source of Ca\(^{2+}\) (Ringer, 1883). Further work demonstrated that in calcium-free solutions, cardiac muscle retains excitability and can initiate and propagate action potentials, although contractility is abolished (Locke and Rosenheim, 1907; Mines, 1913). More recent work demonstrates that the concentration of Ca\(^{2+}\) in extracellular solution determines quantitatively the force of contraction
of cardiac muscle (Reiter, 1964; Jork et al., 1967; Toda, 1969; Landmark, 1972b). The evidence, therefore, indicates that contraction of cardiac muscle is dependent upon an inward displacement of Ca$^{2+}$ across the sarcolemmal complex.

Four routes of entry for Ca$^{2+}$ into myofibers have been identified: 1) Ca$^{2+}$ influx in exchange for K$^+$; 2) Ca$^{2+}$ influx into myofibers by passive diffusion down its electrochemical gradient; 3) Ca$^{2+}$ influx in exchange for Na$^+$ at a sarcolemmal countertransport site; and 4) Ca$^{2+}$ influx through voltage activated, gated ion channels.

Calcium ion entry into myofibers via the first two routes is of very minor importance; the entry of Ca$^{2+}$ in exchange for K$^+$ is thought to be important only in amphibian cardiac muscle, and except under certain pathological conditions, Ca$^{2+}$ entry by passive diffusion is trivial (Nayler and Grinwald, 1981).

Calcium ion entry into cardiac myofibers in exchange for sodium ions, that is, calcium influx via sarcolemmal Na$^+$-Ca$^{2+}$ countertransport, is an established process in mammalian myocardium (Langer, 1982; Reuter, 1982). Early work with isolated cardiac muscle in solutions of varying ionic contents suggested the existence of some sort of "Na$^+$-Ca$^{2+}$ antagonism" which influenced cardiac contraction (Willbrandt and Koller, 1948; Lüttgau and Niedergerke, 1958). Later, $^{45}$Ca$^{2+}$ flux measurements in isolated cardiac
muscle demonstrated that Ca$^{2+}$ efflux was dependent upon extracellular Na$^+$ (Reuter and Seitz, 1967; 1968) and that Ca$^{2+}$ influx was dependent upon intracellular Na$^+$ (Glitsch et al., 1970), leading to the suggestion that an electro-neutral (2 Na$^+$ for 1 Ca$^{2+}$) Na$^+$ - Ca$^{2+}$ countertransport system exists in cardiac sarcolemma (Reuter and Seitz, 1968). Such Na$^+$ - Ca$^{2+}$ exchange is not energy-requiring, but ATP appears to influence the exchange by altering the affinity of the carrier for Na$^+$ or Ca$^{2+}$ or both (Baker and Glitsch, 1973; DiPolo, 1977; Blaustein, 1977). The Na$^+$ - Ca$^{2+}$ exchange process has more recently been demonstrated by $^{45}$Ca$^{2+}$ flux measurements in and out of isolated cardiac sarcolemmal vesicles (Reeves and Sutko, 1979; Pitts, 1979), with some investigators indicating the stoichiometry of Na$^+$ - Ca$^{2+}$ exchange as being greater than 2 Na$^+$ to 1 Ca$^{2+}$ (Reeves and Sutko, 1980; Caroni et al., 1980). While ample evidence is available demonstrating that sarcolemmal Na$^+$ - Ca$^{2+}$ countertransport is a route of entry of Ca$^{2+}$ into cardiac myofibers, there is no evidence suggesting that such an exchange plays an important role in the normal beat-to-beat regulation of myofiber cytosolic Ca$^{2+}$ (Langer, 1980; Nayler and Grinwald, 1981). An enhanced entry of Ca$^{2+}$ in exchange for Na$^+$ may be involved, however, in the response of cardiac muscle to drug interventions which enhance Na$^+$ influx or inhibit the sarcolemmal Na$^+$ pump.
(Akera and Brody, 1981) and in postischemic reperfusion-induced damage (Nayler and Williams, 1978).

Extracellular Ca\textsuperscript{2+} has also been demonstrated to enter cardiac myofibers through sarcolemmal, voltage-activated, gated ion channels. Cardiac myofibers are excitable cells, with electrolytes in cardiac muscle unevenly distributed between the extracellular and intracellular space (Langer, 1968; Trautwein, 1973). Contraction of cardiac myofibers is preceded by sarcolemmal excitation, or a sequence of changes in the ionic permeabilities of the sarcolemma, with different ions carrying charges into and out of the myofiber. This collective sequence of ionic permeability changes is termed an action potential. In a working heart, action potentials are ordinarily initiated in sinoatrial nodal cells and propagated throughout the heart by means of special conducting fibers to the working myocardial cells. While there are only minor ultrastructural differences between working myofibers and those cardiac cells specialized to generate and conduct cardiac action potentials (Bloom and Fawcett, 1975), there are important electrophysiological differences which will be discussed below.

The cardiac action potential may be most simplistically described as a sequential depolarization and repolarization of the myofiber (with the cell normally
possessing an equilibrium voltage of -60 to -90 mV, the inside negative with respect to the outside). The initial depolarizing phase of the action potential has been demonstrated by early workers to involve a large but transient increase in the permeability of the sarcolemma for Na⁺, resulting in a substantial increase in Na⁺ influx (Noble, 1962; Langer, 1967; Reuter and Beeler, 1969a). The repolarization phase has been attributed to a decrease in Na⁺ permeability and an increase in K⁺ permeability, resulting in a K⁺ efflux during repolarization (Fozzard and Gibbons, 1973; Noble, 1975). While the overall shape and character of the cardiac action potential, including a plateau phase between the initial depolarization and repolarization, has been satisfactorily described in terms of different rates of change in Na⁺ and K⁺ permeability (Noble, 1962), the demonstration of a slow inflow of positive ions (a slow component - termed "slow" relative to the initial rapid depolarization due to Na⁺ influx) during the plateau phase has necessitated a modification of this notion. The slow ion flow during the plateau phase has been demonstrated to be an influx of Ca²⁺ or Ca²⁺/Na⁺ which commences when depolarization (due to Na⁺ influx) has reached a certain level (Reuter, 1967; Carmeliet and Vereeke, 1969; Rougier et al., 1969; Reuter, 1975). Hence, the depolarization of the cardiac myofiber is thought to possess two components: an initial, rapid
Na\(^+\) influx (fast current) and a secondary, predominantly Ca\(^{2+}\) influx (slow current); the ions entering the myofiber through separate, voltage-dependent pores or channels in the sarcolemma (Reuter, 1979). In contrast to working myofibers, depolarization in those cardiac cells specialized for action potential generation or conduction is thought to involve Ca\(^{2+}\) influx only (Landmark and Refsum, 1978).

The essential relationship between the occurrence of an action potential and the subsequent contraction of the cardiac myofiber provides suggestive evidence that influx through voltage-dependent ion channels is the primary route of entry of Ca\(^{2+}\) into the myofiber. The demonstration that a proportionality exists between the changes in influx in cardiac muscle during depolarization and changes in contractile force (Winegrad and Shanes, 1962; Langer, 1968; Reuter and Beeler, 1969b; Chapman and Tunstall, 1971) provides more direct evidence that slow current Ca\(^{2+}\) influx during depolarization is the major route of entry for Ca\(^{2+}\) into the cell, and also suggested a source for that Ca\(^{2+}\) which activates contraction in the myofiber. There is a major flaw, however, in the notion that Ca\(^{2+}\) responsible for causing contraction in the cardiac myofiber is supplied to the cytosol exclusively via Ca\(^{2+}\) influx during depolarization. By measuring the dependence
of myofibrillar Ca\(^{2+}\) binding, myofibrillar ATPase activity and isometric tension on free Ca\(^{2+}\) concentration, accurate information has been provided on the amount of Ca\(^{2+}\) required to activate myofilaments to various levels of force development (Solaro et al., 1974). Given this information, it has been recognized that the amount of extracellular Ca\(^{2+}\) entering the cell during depolarization, as measured by integration of slow current during voltage clamp (Beeler and Reuter, 1970; New and Trautwein, 1972), is barely sufficient to produce a force slightly above mechanical threshold. In summary, while cardiac contraction is most certainly dependent upon an influx of extracellular Ca\(^{2+}\) into the cell, and while the cardiac action potential both triggers and serves as a determinant of cardiac contractility by virtue of slow current being the primary route of Ca\(^{2+}\) entry into the cell, that amount of Ca\(^{2+}\) which enters the myofiber during depolarization is by itself insufficient to activate the myofilaments, hence necessitating an additional source or sources of Ca\(^{2+}\) for cardiac contraction.

**Cellular calcium and cardiac contraction**

In view of the evidence presented in the preceding section, a cellular store of Ca\(^{2+}\) which might play a part in the contractile process in cardiac myofibers has been sought. Several myocardial organelles, particularly the
sarcolemmal complex, mitochondria and the sarcoplasmic reticulum, have been proposed to regulate, in some manner, cytosolic Ca\(^{2+}\). It is quite likely that multiple sites may be involved in the regulation of cytosolic Ca\(^{2+}\) in cardiac myofibers.

Ionic exchange studies in which sarcolemmal Ca\(^{2+}\) is displaced with La\(^{3+}\) have provided the basis for one proposal that the sarcolemmal surface coat is the main Ca\(^{2+}\) source for contraction, and that this sarcolemmal Ca\(^{2+}\) is made available to the cytosol during the cardiac action potential by virtue of a nonelectrogenic carrier, which would not appear as a measurable electric current (Langer, 1976). A critical drawback to such a hypothesis, however, is reconciling the electroneutrality of the transport with its dependence upon membrane potential (Kaufmann, 1978). Such a drawback is avoided in a second proposal, which assumes a voltage-dependent release and reuptake of Ca\(^{2+}\) from and by phospholipid moieties at the inner surface of the membrane (Peters, 1978). This proposal, too, has been the target of several criticisms, not the least of which is explaining the presence of Ca\(^{2+}\)-mediated slow inward current in such a hypothetical situation, where rapid depolarization of the myofiber would quickly release a huge store of Ca\(^{2+}\) from the inner surface of the sarcolemma, thus drastically reducing if not reversing the electrochemical gradient for Ca\(^{2+}\) (Kaufmann, 1978). It is
accepted that Ca\textsuperscript{2+} does associate with the sarcolemmal complex (Matsukub et al., 1981; Nayler et al., 1980). It is thought, however, that such Ca\textsuperscript{2+} is in the glycocalyx and is in equilibrium with extracellular Ca\textsuperscript{2+} (Langer, 1978; Nayler and Poole-Wilson, 1981) and, hence, its contribution to cardiac contraction would be via slow current Ca\textsuperscript{2+} influx during depolarization. Sarcolemmal Ca\textsuperscript{2+} probably does not then represent a novel cellular store of the ion.

The role played by mitochondria in the regulation of cytosolic Ca\textsuperscript{2+} in the cardiac myofiber is a controversial one. It is quite clear that mitochondria from various tissues, including cardiac muscle, have the ability to both take up Ca\textsuperscript{2+} from the cytosol (Ca\textsuperscript{2+} uptake being driven electrophoretically by the membrane potential component of the total protonmotive force) and release Ca\textsuperscript{2+} into the cytosol (Carafoli, 1982; Fiskum and Lehninger, 1982). Mitochondria have been demonstrated to adjust their Ca\textsuperscript{2+} influx-efflux cycling in order to buffer the free Ca\textsuperscript{2+} concentration in steady state systems (Fiskum and Lehninger, 1982); such an ability to buffer Ca\textsuperscript{2+} might be of use in lowering the concentration of cytosolic Ca\textsuperscript{2+} during relaxation. The ability of cardiac mitochondria to release Ca\textsuperscript{2+} in response to increased Na\textsuperscript{+} concentration (Carafoli et al., 1974) and cyclic AMP (Borle, 1974) suggests an additional role of mitochondria as intracellular Ca\textsuperscript{2+} stores from which
Ca\(^{2+}\) may be released for contraction. At this point, therefore, mitochondria are intracellular sites which may play roles in both the release of Ca\(^{2+}\) into the cytosol for contraction and the reuptake of Ca\(^{2+}\) from the cytosol for relaxation. Indeed, with the latter being probably more prominent, it has been stated that the affinity of mitochondria for Ca\(^{2+}\), the total binding and storage capacity, and the velocity of Ca\(^{2+}\) uptake processes are "theoretically" adequate to account for relaxation in cardiac muscle (Lehninger, 1974; Carafoli and Crompton, 1978).

The myocardial organelle which is most generally accepted to play the greatest role in cytosolic Ca\(^{2+}\) regulation is probably the sarcoplasmic reticulum (SR). Such an acceptance was originally based, not in small part, upon the role played by skeletal muscle SR in the depolarization-induced release and eventual reuptake of Ca\(^{2+}\) (Adams and Schwartz, 1980). It is established, however, that SR is the major site of subcellular localization of Ca\(^{2+}\) in mammalian myocardium (Legato and Langer, 1969), and that the Ca\(^{2+}\) binding capacity of the SR and the rate at which binding occurs are sufficient to account for both contraction and relaxation in cardiac muscle (Schwartz, 1971). Moreover, it was later indicated (primarily based on evidence from experiments using force development of skinned cardiac muscle as an indicator of Ca\(^{2+}\) availability)
that amount of extracellular (or sarcolemmal) Ca\(^{2+}\) entering the cardiac myofiber during depolarization may induce the release of the store of Ca\(^{2+}\) from cardiac SR which would then activate the myofilaments (Fabiato and Fabiato, 1977). A more direct demonstration of Ca\(^{2+}\)-induced release of Ca\(^{2+}\) from SR of skinned cardiac cells has been reported using a calcium-sensitive fluorescent probe as the indicator of Ca\(^{2+}\) availability (Fabiato and Fabiato, 1979). Additionally, this phenomenon of Ca\(^{2+}\) induced-Ca\(^{2+}\) release from cardiac SR has been demonstrated in isolated cardiac SR vesicles (Katz et al., 1977; Kirchberger and Wong, 1978). A vast body of experimental evidence concerning Ca\(^{2+}\) transport in cardiac SR, the triggering mechanism for release of Ca\(^{2+}\) from SR, the changes in the properties of SR during the cardiac cycle and the modulation of cytosolic Ca\(^{2+}\) by SR during a normal contraction of a cardiac myofiber has been amassed (Winegrad, 1982). This evidence, though not universally accepted (Langer, 1976), indicates that the sarcoplasmic reticulum is most likely the primary intracellular site of regulation for cytosolic Ca\(^{2+}\). Cardiac SR is thought to constitute a storage site for intracellular Ca\(^{2+}\), with that Ca\(^{2+}\) entering the myofiber during depolarization (slow current Ca\(^{2+}\)) proposed to induce or trigger the release of SR Ca\(^{2+}\), which then activates myofilaments to cause contraction. Relaxation, conversely, is believed to be due to
Ca\textsuperscript{2+} uptake back into the cardiac SR. As mentioned above, however, the contribution of mitochondria to the regulation of cytosolic Ca\textsuperscript{2+} during cardiac contraction and relaxation cannot be excluded.

**Protein phosphorylation and cardiac contractility**

Calcium ion is of primary importance in regulating muscle contraction, and the primary means by which regulation is imposed upon cardiac myofiber contraction is through Ca\textsuperscript{2+} binding to the troponin-tropomyosin complex. Additional regulation of cardiac contraction is provided via phosphorylation of proteins at several sites in the myofiber. (Adelstein and Eisenberg, 1980; Barany and Barany, 1981; Tada and Katz, 1982).

Phosphorylation of cardiac troponin I by a cyclic AMP-dependent protein kinase has been demonstrated by several investigators (Reddy et al., 1973; England, 1975; Solaro et al., 1976; Ezrailson et al., 1977). Furthermore, it has been demonstrated that when cardiac troponin I is phosphorylated, higher than normal Ca\textsuperscript{2+} concentrations are required for activation of myosin-ATPase (Barany and Barany, 1980), that is, phosphorylation of troponin I decreases the Ca\textsuperscript{2+} sensitivity of the actin-myosin interaction. It is currently thought that this decrease in sensitivity is due to a reduction in the affinity of troponin C for Ca\textsuperscript{2+} when troponin I is phosphorylated.
Solaro et al., 1981). It has been speculated that this cyclic AMP-induced phosphorylation of troponin I plays an important role in the acceleration of relaxation in the response of cardiac muscle to positive inotropes which stimulate adenylate cyclase (Katz, 1980).

The phosphorylation of a second myofibrillar protein, a 150,000 dalton myosin-associated protein termed the C protein, has also been reported in cardiac muscle (Jeacocke and England, 1980). This protein is phosphorylated by the same kinase as troponin I, and is indeed phosphorylated in parallel with troponin I in the catecholamine-treated heart. The functional significance of the phosphorylation of this protein, however, has not yet been made clear.

Myosin light chain phosphorylation is the primary means of regulation of the actin-myosin interaction in smooth muscle, and may play a role in fine modulation of contraction in cardiac muscle (Adelstein and Eisenberg, 1980; Barany and Barany, 1981). Phosphorylation of the light chains of myosin is thought to be a prerequisite for actin-activation of myosin-ATPase in those cells which are regulated primarily through light chain phosphorylation. Ca^{2+} regulates this system by being required for the activation of myosin light chain kinase—a highly specific, cyclic nucleotide-independent, Ca^{2+}-requiring enzyme which catalyzes the phosphorylation of the myosin light chains.
Myosin light chain kinase in its active form is composed of two proteins; the first being a heavy chain of variable molecular weight, and the second being the smaller Ca$^{2+}$-binding protein calmodulin. The binding of Ca$^{2+}$ to calmodulin activates this kinase, which phosphorylates the myosin light chains, resulting in a form of myosin that can undergo contraction. Dephosphorylation of the light chains by a second enzyme, myosin light chain phosphatase, restores myosin to a form that cannot be activated by actin. As is the case with the actin-based regulation of contraction in cardiac muscle, Ca$^{2+}$ plays a critical role in the regulation of smooth muscle contraction by virtue of its requirement for the activation of myosin light chain kinase activity. A second Ca$^{2+}$-independent mechanism for regulating myosin light chain kinase activity involves the phosphorylation of the light chain kinase by a cyclic AMP-dependent protein kinase. The phosphorylation of the light chain kinase results in decreased kinase activity apparently due to a decrease in the binding constant of the phosphorylated kinase for the Ca$^{2+}$-calmodulin complex (Adelstein and Eisenberg, 1980). Myosin light chain phosphorylation has been suggested as a possible modulator of cardiac contraction based upon the important role played by this regulatory system in many noncardiac cells, and the presence of the enzyme myosin light chain kinase
which was shown to be activated in the presence of $\text{Ca}^{2+}$ and calmodulin, in cardiac muscle (Walsh et al., 1979; Wolf and Hofmann, 1980). At present, the case for a major role of this system in regulating cardiac contraction is not very strong. Most investigators agree that the actin-myosin-activation of cardiac myosin-ATPase does not require myosin phosphorylation (Morgan et al., 1976; Perry, 1979; Stull et al., 1980). Furthermore, a good correlation between myosin light chain phosphorylation and contractility is generally lacking in cardiac muscle, as is the presence of the enzyme myosin light chain phosphatase (Barany and Barany, 1981). Pending future investigation, however, myosin light chain phosphorylation cannot be absolutely excluded as a possible modulator of cardiac contraction.

Aside from myofilaments, there are two additional sites of protein phosphorylation in the cardiac myofiber, sarcoplasmic reticulum and sarcolemma (Barany and Barany, 1981; Tada and Katz, 1982). The particular site and functional significance of phosphorylation is better understood for the former structure.

As noted in the preceding section, sarcoplasmic reticulum (SR) is thought to be an important structure for the regulation of cytosolic $\text{Ca}^{++}$. Cardiac SR is thought to release intracellular $\text{Ca}^{2+}$ (in response to extracellular $\text{Ca}^{2+}$ influx) in order to activate the myofilaments during
contraction; and the cardiac SR is thought to actively extract Ca\(^{2+}\) out of the cytosol in order to induce relaxation (a reduction in cytosolic Ca\(^{2+}\) favoring displacement of Ca\(^{2+}\) from the troponin-tropomyosin complex, resulting in a renewed hindrance of actin-myosin interaction), the latter process due primarily to an SR-Ca\(^{2+}\)-ATPase. A cyclic AMP-dependent protein kinase-catalyzed phosphorylation of an SR membrane-bound protein, called phospholamban, has been found to be responsible for an accelerated Ca\(^{2+}\) uptake by SR (Toda et al., 1976). The molecular mechanism by which phospholamban phosphorylation results in an enhanced uptake of Ca\(^{2+}\) into SR has been elucidated as a result of a great amount of work done by numerous investigators (Tada and Katz, 1982). Most simplistically phosphorylation of phospholamban by cyclic AMP-dependent protein kinase confers an increased Ca\(^{2+}\) sensitivity to the SR-Ca\(^{2+}\)-ATPase, so that Ca\(^{2+}\) uptake may occur at lower Ca\(^{2+}\) concentrations and the rate of uptake is accelerated. This cyclic AMP-dependent acceleration of SR Ca\(^{2+}\) uptake might play a role in the acceleration of relaxation in the response of cardiac muscle to positive inotropic agents which stimulate adenylate cyclase (Katz, 1980). Of far more general functional significance, however, is the discovery that phospholamban may be phosphorylated not only by the cyclic AMP-dependent protein kinase, but also by a
Ca\(^{2+}\)-calmodulin-dependent cardiac SR—bound protein kinase (Le Peuch et al., 1979). The Ca\(^{2+}\)-calmodulin-dependent phosphorylation of phospholamban also accelerates Ca\(^{2+}\) uptake into cardiac SR (Lopashuk et al., 1980; Kranius et al., 1980), and has been proposed to be the major regulator of Ca\(^{2+}\) flux at the SR in normal cardiac muscle (Barany and Barany, 1981). Hence, in addition to extracellular Ca\(^{2+}\) triggering the release of additional SR Ca\(^{2+}\) to activate myofilaments in causing contraction, high levels of cytosolic Ca\(^{2+}\) probably stimulate Ca\(^{2+}\) removal from the cytosol by means of SR phosphorylation to cause relaxation.

The status and physiological significance of sarcolemmal phosphorylation are more controversial than those of SR phosphorylation; the controversy due, not in small part, to the difficulties in obtaining pure, functional sarcolemma to study. It has been fairly well documented by several groups using different experimental systems that one or more sarcolemmal proteins are phosphorylated by a cyclic AMP-dependent protein kinase, with the consensus being that two sarcolemmal peptides (9000-16,000 dalton and 22,000-24,000 dalton) serve as major substrates for cyclic AMP-dependent phosphorylations, with several proteins of higher weights serving as minor substrates (Tada and Katz, 1982). It has been suggested that phosphorylation of sarcolemmal preparations by a cyclic AMP-dependent protein
kinase may be augmenting the activity of the sarcolemmal-
Ca^{2+}-ATPase enzyme (Hui et al., 1976; Lamers et al., 1981). More recently, it has been demonstrated that the sarcolemmal Ca^{2+}-ATPase is also regulated by Ca^{2+}-calmodulin-activated phosphorylation, with such Ca^{2+}-dependent phosphorylation augmenting the activity of the enzyme (Caroni and Carafoli, 1981; Tuana et al., 1981; Kuwayama & Kanazawa, 1982). As in the case of the dual regulation of cardiac SR-Ca^{2+}-ATPase, the augmentation of sarcolemmal Ca^{2+}-ATPase activity by cAMP-dependent phosphorylation may play a role in the acceleration of relaxation in the response of cardiac muscle to positive inotropes which stimulate adenylate cyclase, while the augmentation of enzyme activity by Ca^{2+}-calmodulin-dependent phosphorylation may be of greater overall functional significance in the process of relaxation in normal cardiac muscle. One final aspect of sarcolemmal protein phosphorylation and the regulation of cardiac contractility bears mentioning in this section. It is well established that the slow inward Ca^{2+} current which occurs during the cardiac action potential is modulated by cyclic AMP, such that agents which stimulate adenylate cyclase increase the plateau height and duration of action potential—indications of an augmented Ca^{2+} influx through the sarcolemmal channels or pores (Reuter, 1974). Several models have been advanced, attributing the cyclic
AMP-dependent augmentation of slow channel Ca\(^{2+}\) influx to the cAMP-dependent phosphorylation of a sarcolemmal protein, which would result in the formation of new Ca\(^{2+}\) channels, the activation of dormant, "dephospho" Ca\(^{2+}\) channels or an increase in the Ca\(^{2+}\) affinity of the Ca\(^{2+}\) channels (Speralakis and Schneider, 1976; Wollenberger and Will, 1978; Reuter, 1979). It has also been speculated that calmodulin may be involved in the gating of the slow inward Ca\(^{2+}\) current (Johnson et al., 1981). Future work is needed, however, to define the relationship, if any, between sarcolemmal protein phosphorylation and slow inward Ca\(^{2+}\) current (Tada and Katz, 1982).

**Calcium antagonism**

Calcium antagonism, an interference with the action and/or availability of Ca\(^{2+}\) in a cell, was first introduced as a pharmacodynamic property to describe the cardiodepressant actions of certain pharmacological agents. The term "calcium antagonist" was introduced on the basis of the following four separate observations: a) Ca\(^{2+}\) reverses the cardiodepressant effect of verapamil and D600; b) verapamil, D600 and nifedipine depress Ca\(^{2+}\)-induced tension development in K\(^{+}\)-depolarized cardiac muscle; c) despite the absence of beta-adrenoceptor blocking activity, these drugs prevent the myocardium from becoming overloaded with Ca\(^{2+}\) in response to large amounts of isoproterenol, which
augments Ca$^{2+}$ slow current influx; and d) these drugs selectively inhibit the Ca$^{2+}$-dependent component of the cardiac action potential (Fleckenstein et al., 1969; Fleckenstein, 1970; Fleckenstein et al., 1972).

By virtue of their utility as experimental tools and potential therapeutic agents, the "calcium antagonists" have, in recent years, been the focus of massive scientific interest and investigation. As a result, some degree of a better understanding of calcium antagonism as a pharmacodynamic property has emerged. It is now recognized that a wide variety of agents may be designated as "calcium antagonists" based upon their ability to inhibit Ca$^{2+}$-dependent processes with varying potencies and specificities (Rahwan et al., 1979b). It is also recognized that the term "calcium antagonist," which lacks specificity with respect to a precise site of action, and misleadingly implies a classical receptor-agonist-antagonist relationship, does not provide an adequate description of the pharmacodynamic properties of the heterogeneous group of molecules which are "calcium antagonists" (Nayler and Poole-Wilson, 1981).

"Calcium antagonists" have been classified into two major groups by virtue of their predominant site of action (Rahwan, 1982). The compounds which comprise the first group, which are thought to act primarily by inhibiting
the influx of extracellular $\text{Ca}^{2+}$ into cells via the slow current during the action potential, have been designated as calcium entry blockers, slow calcium channel blockers or slow calcium channel inhibitors (Nayler and Grinwald, 1981; Nayler and Poole-Wilson, 1981; Rahwan, 1982). This group encompasses the majority of compounds which have been formerly loosely termed "calcium antagonists," and is exemplified by the compounds verapamil, nifedipine and diltiazem (Fleckenstein, 1977; Rosenberger and Triggle, 1978). It should be noted, however, that agents which are collectively designated as calcium entry blockers are structurally heterogeneous, usually possess other pharmacodynamic properties other than calcium entry blockade, probably possess different membrane sites of action for calcium entry blockade and may indeed inhibit $\text{Ca}^{2+}$-dependent processes by virtue of other "calcium antagonistic" mechanisms as well as through blockade of slow calcium currents (Nayler and Grinwald, 1981; Nayler and Poole-Wilson, 1981; Rahwan, 1982). Several calcium entry blockers have been found to be of considerable therapeutic value, particularly in the management of cardiovascular diseases, including exertional angina, variant angina, arrhythmias, hypertension, hypertrophic cardiomyopathy, pulmonary hypertension and myocardial infarction (Antman et al., 1980; Stone et al., 1980). Their utility in the therapy of these disorders is generally ascribed to their ability to inhibit
slow current \( \text{Ca}^{2+} \) entry, although additional pharmacological actions may contribute to their therapeutic usefulness.

The compounds which comprise the second group of "calcium antagonists," which are thought to exert their pharmacological actions by blocking intracellular \( \text{Ca}^{2+} \) receptors, preventing intracellular \( \text{Ca}^{2+} \) mobilization, facilitating \( \text{Ca}^{2+} \) sequestration by intracellular organelles or enhancing \( \text{Ca}^{2+} \) efflux from the cell, have been designated as intracellular calcium antagonists. This group of "calcium antagonists" is exemplified by the trimethoxybenzoate compounds (Malagodi and Chiou, 1974; Chiou and Malagodi, 1975) and the methylenedioxyindenes (Rahwan and Witiak, 1982).

**Pharmacology of selected 2,3-substituted-5,6-methylenedioxyindenes**

A series of 2-substituted 3-dimethylamino-5,6-methylenedioxyindenes (MDIs) were synthesized by Witiak et al. (1974). The following discussion is a review of the relevant pharmacology of two key analogues in the original series of MDIs, the 2-\( \text{n} \)-propyl and 2-\( \text{n} \)-butyl MDI hydrochlorides (pr-MDI and bu-MDI, respectively), as well as that of the quaternary ammonium analogue of bu-MDI, Q-bu-MDI (2-\( \text{n} \)-butyl-5,6-methylenedioxyindene-3-trimethylammonium iodide) (see Fig. 1 for structures). A more comprehensive review of the chemistry, pharmacology and toxicology of the
Figure 1. Chemical structures of propyl- (pr-), n-butyl- (bu-) and quaternary n-butyl- (Q-bu-) aminomethylene-dioxyindenes (MDIs).
pr-MDI

bu-MDI

Q-bu-MDI

Fig. 1
MDI compounds, including structural analogues, is available elsewhere (Rahwan and Witiak, 1982).

The pr- and bu-MDIs were initially evaluated in uterine smooth muscle. In estrogenized rat uterus, the pr- and bu-MDIs blocked the spasmogenetic action of PGF$_{2\alpha}$, PGE$_2$, oxytocin, barium, acetylcholine and ergonovine in concentration-dependent and reversible manners. It was noted, in the rat uterus, that the antagonism by these MDIs of the spasmogenetic actions of acetylcholine [which utilizes extracellular Ca$^{2+}$ (Rubin, 1970; Daniel and Janis, 1975)] and barium [which utilizes intracellular Ca$^{2+}$ (Saito et al., 1972; Antonio et al., 1973)] were both reversed with increasing concentrations of extracellular Ca$^{2+}$ (Rahwan et al., 1977). In GI smooth muscle, the pr- and bu-MDIs blocked the contractile effect of histamine on the isolated guinea pig ileum, and of acetylcholine on the isolated rat ileum (Rahwan et al., 1977). In vascular smooth muscle, the pr- and bu-MDIs produced prolonged, concentration-dependent relaxations of K$^+$-depolarized strips of bovine coronary vessels, which were reversible upon elevation of extracellular Ca$^{2+}$ (Piascik et al., 1979a); while in rat aorta, in the absence of extracellular Ca$^{2+}$, the pr- and bu-MDIs inhibited the contractile effects of U44069 (a stable analogue of PGH$_2$) and norepinephrine, respectively (Heaslip and Rahwan, 1982a; 1982b). In the absence of
extracellular Ca\(^{2+}\), the contractile effects of U44069 (Loutzenhiser and Van Breeman, 1981) and of norepinephrine (Heaslip and Rahwan, 1982c) are thought to be due to mobilization of intracellular Ca\(^{2+}\).

The pr- and bu-MDIs have been evaluated in two skeletal muscle systems. In the isolated rat diaphragm, pr-MDI blocked caffeine-induced contractures both in the presence and in absence of extracellular Ca\(^{2+}\) (Rahwan and Gerald, 1981). Caffeine-induced contractures in skeletal muscle are thought to be mediated by the mobilization of intracellular Ca\(^{2+}\) from sarcoplasmic reticulum (Bianchi, 1975). In the isolated frog sartorius muscle, bu-MDI depressed activation heat upon stimulation (Burchfield et al., 1982)—activation heat represents the energy liberated in association with Ca\(^{2+}\) mobilization and sequestration in contracting muscle (Homsher et al., 1972; Smith, 1972).

To further characterize the pharmacological properties of the pr- and bu-MDIs, the compounds were evaluated in two models of stimulus-secretion coupling. In the isolated, perfused bovine adrenal medulla, the pr- and bu-MDIs reversibly blocked catecholamine secretion evoked by carbachol, but did not affect acetaldehyde-induced catecholamine secretion (Piascik et al., 1978). Adrenal catecholamine secretion induced by the former agent is known to be mediated by extracellular Ca\(^{2+}\) (Schneider, 1969).
and not by intracellular Ca$^{2+}$ (Borowitz, 1969), while acetaldehyde-induced secretion is Ca$^{2+}$-independent (Schneider, 1971; Rahwan et al., 1974; O'Neill et al., 1975). Furthermore, in the bovine adrenal medulla, the pr- and bu-MDIs did not affect $^{45}$Ca$^{2+}$ uptake by chromaffin cells (Piascik et al., 1978). The effect of pr-MDI upon platelet aggregation, which is thought to involve a stimulus-induced translocation of intracellular Ca$^{2+}$ which in turn causes the release of granular contents (Feinstein, 1978), is two-fold. At concentrations up to 0.5 mM, pr-MDI induced the platelet release reaction, while at 0.5 mM and greater, pr-MDI inhibited the thrombin-induced platelet release reaction (Müller et al., 1981).

Prior to the commencement of the studies described in the body of this dissertation, some information on the direct effects of the MDIs upon cardiac muscle was available. It had been reported that histamine-activated cardiac adenylate cyclase, which has the properties of a histamine-2 ($H_2$) receptor, is blocked by pr-MDI ($pA_2 = 6.46$) and various other calcium entry blockers, as well as by the specific $H_2$ antagonist cimetidine ($pA_2 = 6.1$) (Johnson and Grupp, 1979). The significance of this finding is not clear, however, in that this $H_2$ blocking property of the "calcium antagonists" corresponds neither to their calcium antagonistic properties nor to their ability to block the positive inotropic action of histamine (Johnson and Grupp,
1979). In the nonstimulated, isolated perfused rabbit heart preparation, both the pr- and bu-MDIs produced negative inotropic effects and increased coronary flow, with no alteration in chronotropy (Piascik et al., 1979a). Finally, in an initial evaluation of the antiarrhythmic properties of the MDIs, it was found that ouabain-induced ventricular arrhythmias in dogs were reversed by intravenous administration of either pr- or bu-MDI, the reversion being preceded by a drop in diastolic blood pressure, with no appreciable alteration in systolic pressure or heart rate. Furthermore, pretreatment of dogs with pr- or bu-MDI afforded significant protection against ouabain-induced arrhythmia (Piascik et al., 1979b).

Collectively, the pharmacological studies cited above suggested that the pr- and bu-MDIs might be acting as "calcium antagonists" with an intracellular site of action. Support for such a mechanism is derived from the following findings (summarized from above): (1) their nonselective spasmolytic activity in nonvascular smooth muscle, including their ability to interfere with barium-induced (intracellular Ca^{2+}-dependent) smooth muscle contraction; (2) the reversibility of their vascular and nonvascular smooth muscle relaxant properties by increasing extracellular Ca^{2+}; (3) their ability to inhibit Ca^{2+}-dependent (but not Ca^{2+}-independent) evoked adrenomedullary catecholamine secretion
without blocking Ca\(^{2+}\) entry into the cell; (4) their inhibitory effects on caffeine-induced (intracellular Ca\(^{2+}\)-dependent) contracture of skeletal muscle in both the presence and absence of extracellular Ca\(^{2+}\); (5) their ability to depress the activation heat in skeletal muscle (which reflects a reduced mobilization of Ca\(^{2+}\) from intracellular stores); (6) their inhibitory effect on thrombin-induced platelet secretion, which is intracellular Ca\(^{2+}\)-dependent; and (7) their ability to block the intracellular Ca\(^{2+}\)-dependent contractile effects of norepinephrine and U44069 on the isolated rat aorta in calcium-free medium.

Several independent studies, most of which were conducted concurrently with those studies described in the bulk of this dissertation have sought to more fully elucidate the mechanism of action of the pr- and bu-MDIs or, in most cases, identify possible intracellular sites of action of the compounds. The electrophysiologic and mechanical effects of the pr- and bu-MDIs, as well as those of Q-bu-MDI (the quaternary ammonium analogue of bu-MDI) have been investigated in isolated, superfused canine Purkinje and ventricular muscle fibers, using standard microelectrode techniques. The electrophysiologic changes in these cardiac muscle preparations (reviewed in greater detail in Rahwan et al., 1981; Muir, 1982) indicated that all three analogues possessed the ability to interfere with both the fast (Na\(^{+}\)-dependent) and slow (Ca\(^{2+}\)-dependent) components of the
cardiac action potential. The latter observed property of the compounds, was further demonstrated in Purkinje fibers in which fast (Na\(^+\)-dependent) current was abolished (with tetrodotoxin, lidocaine or superfusion with Na\(^+\)-free solution) and in which slow (Ca\(^{2+}\)-dependent) current is augmented (with a catecholamine) (Rahwan et al., 1981; Muir, 1982). However, it was noted in the isolated superfused canine papillary muscle preparation that the pr- and bu-MDIs were able to uncouple excitation-contraction coupling at concentrations which did not alter action potential characteristics (Muir, 1982). This study, therefore, indicates that the pr-, bu- and Q-bu-MDI interfere with transmembrane electrical activity in canine cardiac muscle, yet may also operate through an additional mechanism (separated and unrelated to the interference with ion fluxes at the sarcolemma) to account, in some part, for the depression in myocardial contractility.

The remaining studies have sought to focus upon the possible intracellular site or sites of action of the MDIs. It has been reported that bu-MDI binds to brain calmodulin, resulting in inhibition of Ca\(^{2+}\)-calmodulin-dependent phosphodiesterase activity, and that bu-MDI also binds to cardiac troponin C (Piascik et al., 1981). A recent report (Weishaar et al., 1982) contradicts the findings of the preceding report regarding an MDI effect upon calmodulin, and similarly does not support an effect of the MDIs upon
sarcoplasmic reticulum, which is indicated in skeletal muscle studies (Rahwan and Gerald, 1981; Burchfield et al., 1982). Using a variety of isolated cardiac subcellular systems, these investigators have reported that at concentrations that depress myocardial contractility, the bu-MDI has no effect upon $\text{Ca}^{2+}$ uptake, transport or release by cardiac sarcoplasmic reticulum or mitochondria, nor does the compound affect the intracellular $\text{Ca}^{2+}$-binding protein calmodulin; but rather that bu-MDI depresses contractility by interfering with electron transport or uncoupling oxidative phosphorylation in mitochondria (Weishaar et al., 1982). This hypothesis is not supported, however, by an earlier study concerning the effects of the pr- and bu-MDIs upon mitochondrial function, which reported that the compounds preserve mitochondrial function and structure in the presence of high inorganic phosphate concentrations, which induce mitochondrial swelling and uncoupling of oxidative phosphorylation (Matlib et al., 1981).

The electrophysiological and molecular studies reviewed above suggest several possible sites of action for the MDIs, including several intracellular sites for the pr- and bu-MDIs, any one of which might serve as a site at which the action of $\text{Ca}^{2+}$ or its availability to the rest of the cell might be altered. Clearly, however, a consensus for any particular site or specific mechanism is, at this point, still lacking.
Statement of the Problem

Extensive evidence suggests that the two tertiary amine MDIs, pr- and bu-MDI, act as "calcium antagonists" with a possible intracellular site of action. When the studies described in this dissertation were commenced, relatively little information was available regarding the direct effects of these compounds on cardiac muscle, a physiological system in which the role of Ca$^{2+}$ (particularly with regard to contractility) is, to some degree, understood.

It was proposed, therefore, to examine the pr- and bu-MDIs in several cardiac systems, which would allow not only for an evaluation of the type of pharmacological effects produced by the compounds, but would also provide information regarding the possible sites or mechanisms of action by which the compounds might be interfering with Ca$^{2+}$ availability and/or action. The quaternary ammonium Q-bu-MDI was also examined in several systems to provide some insight about extracellular vs intracellular sites of action. By virtue of its charge, the Q-bu-MDI is less likely to cross biological membranes than its uncharged tertiary analogues, and would be confined, in great part, to the extracellular space. The studies proposed in this dissertation may be classed into three major groups: in vivo antiarrhythmic studies, in vitro studies in isolated
atria, and in vitro studies on subcellular localization of the MDIs in cardiac tissue.

To extend the antiarrhythmic profiles of the pr- and bu-MDIs, and to evaluate the activity of Q-bu-MDI, the study of several models of experimental arrhythmia in several animal species was undertaken. Models in which arrhythmias are induced by excess Ca$^{2+}$ in dogs and rats, aconitine in rats, methacholine in rats and chloroform/hypoxia in mice allow for evaluation of the ability of compounds to protect against dysrhythmias which vary widely in electrophysiologic/ionic origin.

Three phases of in vitro studies in isolated atria were undertaken. The effects of the tertiary and quaternary MDIs upon the mechanical and electrical activities of cardiac muscle (inotropy, chronotropy, frequency-force relationship and threshold voltage) were examined in spontaneously beating right and electrically-paced guinea pig left atria. Compounds which are classed as "calcium antagonists", particularly calcium entry blockers, display characteristic, well documented effects upon rate, force and frequency-force relationship in such systems. The second proposed phase of studies in isolated atria was of a more mechanistic nature, and involved the evaluation of the ability of positive inotropic interventions (excess Ca$^{2+}$, catecholamine, cardiac glycoside) to reverse the negative inotropic effect of the tertiary MDIs, and conversely, to evaluate
the effect of the MDIs upon concentration-response relationships mediated by these interventions. Both studies were conducted in electrically-paced guinea pig left atria. The former evaluation would establish whether the MDIs meet a key criterion set forth by Fleckenstein (1977) for classification of agents as "calcium antagonists," while the latter evaluation would provide information regarding a possible membrane calcium entry blocking property of the MDIs as has been demonstrated for the calcium channel blockers verapamil and D600 (Bristow and Green, 1977). The final proposed phase of isolated atrial studies, again conducted in electrically-paced guinea pig left atria, was designed specifically to test the hypothesis (Weishaar et al., 1982) that the MDIs depress myocardial contractility via an interference with energy production by mitochondria. This latter evaluation involved a comparison of the reversibility by excess Ca\(^{2+}\) or catecholamine of the comparable cardiodepressions induced by the MDIs and by agents which are known to interfere with mitochondrial energy production (uncouplers of oxidative phosphorylation and inhibitors of electron transport).

Finally, in vitro studies of the uptake, washout, tissue concentration and subcellular distribution of pr-MDI were undertaken to address some basic issues
involved in the concept of an intracellular site of action of the compound. The radiolabelling of pr-MDI in the indene ring was a prerequisite for these studies.
CHAPTER II

MATERIALS AND METHODS

Evaluation of MDIs in In Vivo Models of Arrhythmia

Calcium-induced arrhythmias in dogs

Dogs of either sex (Blue's Animal Farm, Plain City, OH), weighing 8-13.5 kg, were anesthetized with sodium pentobarbital. The right radial arm vein was cannulated for drug administration. When necessary, a tracheal cannula was inserted to assist respiration. Electrocardiogram (Lead II) was monitored via a Physiograph preamplifier (MK III, E and M Instrument Company, Houston, Texas) and recorded on a Physiograph IV recorder (E and M Instrument Company). The animals were pretreated with either pr-MDI or bu-MDI or with 5% dextrose (as control). The MDIs were dissolved in 5% aqueous dextrose solution, and administered in the right radial arm vein in a volume of 10 ml and a rate of 2 ml/min. Fifteen minutes after completion of the pretreatment, a continuous infusion of calcium chloride solution (500 mg/kg/hr CaCl₂·2H₂O) was begun in the cannulated arm vein using a syringe pump (Model 901, Harvard Apparatus Company, Mills, Mass.), and maintained until the
animals died. The times to onset of radical arrhythmias and to death were recorded, as were the type and severity of deviations from the normal ECG.

Calcium-induced arrhythmias in rats

Male, Sprague-Dawley rats (Lab Supply Company, Indianapolis, Ind.), weighing 150-220 g, were anesthetized with sodium pentobarbital. The left jugular vein was cannulated for drug administration. Electrocardiogram (Lead II) was monitored via a Physiograph MK III preamplifier and recorded on a Physiograph IV recorder. The animals were pretreated with one of the following drugs: pr-MDI, bu-MDI, Q-bu-MDI, and, for comparative purposes, prenylamine (Segontin, Hoechst-Roussel Pharmaceuticals, N. Sommerville, N.J.), verapamil (Isoptin, Knoll Pharmaceutical Company, Whippany, N.J.), diphenylhydantoin (Dilantin, Parke-Davis and Company, Detroit, Mich.), quinidine (Quinidine Gluconate Injection USP, Eli Lilly and Company, Indianapolis, Ind.) or appropriate vehicle (control). With the exceptions of quinidine and diphenylhydantoin, all drugs were dissolved in 5% aqueous dextrose solution. Quinidine was provided in a vehicle of sterile water containing 0.005% edetate disodium and 0.25% phenol, which was diluted with 5% aqueous dextrose to the appropriate dose. Diphenylhydantoin was dissolved in the standard vehicle of 40% propylene glycol and 10% ethanol in distilled water and
diluted to the appropriate dose (pH 12). All drugs were administered into the jugular vein over a 40-second period. Ten minutes following pretreatment, an acute dose of 1 ml/kg of a 10% CaCl$_2$·2H$_2$O solution was injected into the jugular vein over a period of 10 seconds. The arrhythmias resulting from the calcium administration were graded according to: (a) initial change in heart rate following calcium administration; (b) incidences of sino-atrial block, atrio-ventricular block (2nd or 3rd degree), ectopic beats (nodal and ventricular, single and multiple), and ventricular flutter and fibrillation; (c) time required for reversion to control ECG; and (d) incidence of mortality.

Aconitine-induced arrhythmias in rats

The antiarrhythmic activities of the MDIs (pr-, bu- and Q-bu-MDI) and quinidine were evaluated according to the method of Haas and Busch (1968). Male Sprague-Dawley rats weighing 180-250 g, were anesthetized with sodium pentobarbital. The left jugular vein was cannulated for drug administration, and the ECG Lead II was continuously monitored. The antiarrhythmic compound was injected into the jugular vein 5 min before the start of an intravenous infusion of 10 µg/kg/min of aconitine delivered at a constant rate of 0.136 ml/min. The onset of ventricular arrhythmias, which usually consisted of a series of downward deflections with enlarged QRS complexes lasting 5 sec
or more (Vargaftig and Coignet, 1969), was chosen as the end point. The results are expressed in µg/kg of aconitine required to precipitate ventricular arrhythmias in the presence or in the absence of antiarrhythmic pretreatment. The antiarrhythmic agents were delivered in a vehicle of 5% dextrose, while aconitine was dissolved in distilled water to which a few drops of 2 N HCl had been added. Unprotected controls were treated with 5% dextrose.

**Methacholine-induced arrhythmias in rats**

The protocol employed in this evaluation of the antiarrhythmic activities of the MDIs (pr-, bu- and Q-bu-MDI) and quinidine was similar to that described in the preceding section for aconitine-induced arrhythmias. The antiarrhythmic agent was injected into the jugular vein 5 min before starting an intravenous infusion of 80 µg/kg/min of acetyl-β-methylcholine chloride (methacholine) delivered at a constant rate of 0.136 ml/min. The ECG was monitored continuously until the onset of a consistent, characteristic atrioventricular dissociation which was considered the end-point. The results are expressed in µg/kg of methacholine required to induce atrioventricular dissociation in the presence or in the absence of antiarrhythmic pretreatment. The antiarrhythmic agents were delivered in a 5% dextrose vehicle, while methacholine chloride was dissolved in distilled water (pH 4). Unprotected controls were treated with 5% dextrose.
Chloroform/hypoxia-induced arrhythmias in mice

The chloroform inhalation procedure described by Lawson (1968) was used to evaluate the antiarrhythmic properties of the MDIs (pr-, bu- and Q-bu-MDI) and quinidine. In this assay, chloroform inhalation leading to respiratory arrest produces ventricular arrhythmia. Female Swiss albino mice, weighing 16-22 g, were pretreated intraperitoneally with the antiarrhythmic agent (dissolved in 5% dextrose) 10 min before exposure to chloroform in a closed container containing chloroform-saturated cotton. The mice were removed from the chloroform atmosphere immediately after respiratory arrest, and the Lead II ECG monitored. The antiarrhythmic ED50 values with their 95% confidence limits were calculated for the antiarrhythmic agents by the standard method of Litchfield and Wilcoxon, as were the slope functions and tests for parallelism of antiarrhythmic dose-response curves. In a separate experiment, the 24-hr LD50 of Q-bu-MDI was determined.

Statistical analysis for antiarrhythmic studies

Values are presented as mean ± S.E.M. unless otherwise indicated. Statistical comparisons of arrhythmogenic doses of aconitine and methacholine in the presence and absence of antiarrhythmic pretreatments; and of times to onset of arrhythmia, times to death, ECG reversion times and chronotropic alterations in experiments involving
arrhythmias induced by Ca\(^{2+}\) were done using an analysis of variance for a one-way randomized design. Statistically different F-values were then tested using the Dunnett test for multiple comparisons (Winer, 1971). The statistical method of Litchfield and Wilcoxon (1949) was used to determine antiarrhythmic ED50s, LD50s, and statistical differences between pretreatments. In all cases, the level of significance was set at p < 0.05.

**Evaluation of the MDIs in Isolated Guinea Pig Atrial Preparations**

**Effects upon mechanical and electrical activity**

English short-haired guinea pigs (Carr Animal Supply, Powell, Ohio) of either sex, weighing 300-600 g, were killed by a blow on the head. The right and left atria were quickly removed by dissection and suspended in a water-jacketed organ bath containing Krebs-Henseleit solution bubbled with 95% O\(_2\) and 5% CO\(_2\) at 32°C. The composition of the physiological solution (g/l) was as follows: NaCl 6.92; KCl 0.35, MgSO\(_4\)\(\cdot\)7H\(_2\)O 0.29, CaCl\(_2\)\(\cdot\)2H\(_2\)O 0.37, KH\(_2\)PO\(_4\) 0.16, NaHCO\(_3\) 2.1, dextrose 2.1. Contractile force was recorded isometrically under 500 mg preload, using a Narco Biosystems force transducer (E and M Instrument Company, Houston, Texas) connected to a physiograph DMP-4B recorder (E and M Instrument Company).
Isolated left atria were electrically driven by square wave impulses of 3 msec duration at a frequency of 3 Hz with twice the threshold voltage, delivered by a Grass S9 stimulator (Grass Instrument Company, Quincy, Mass.). The atria were stimulated with two platinum electrodes, one electrode (anode) being in direct contact with the tissue, while the second electrode, a ring electrode (cathode), being suspended 2.5 cm above the anode. An equilibration time of one hour was allowed before redetermination of threshold voltage and generation of predrug (control) frequency-force profile in the range of 1-5 Hz. Following re-equilibration at 3 Hz with twice the threshold voltage, one cumulative dose-response curve (contractile force) for one MDI analogue was obtained, followed immediately by redetermination of threshold voltage and generation of post-drug frequency-force profile. Sustained peak effect for a given concentration of MDI analogue, in the range of $10^{-8}$ M to $3 \times 10^{-4}$ M, was observed within 3 to 5 minutes of addition to the organ bath. The mean time courses for generation of the entire dose-response curve for each of the MDI analogues, including the generation of predrug and postdrug frequency-force profiles, were (minutes, mean ± SEM): bu-MDI (55.0 ± 3.9), Q-bu-MDI (56.9 ± 1.5) and pr-MDI (60.8 ± 4.2).

Spontaneously beating right atria were also allowed a one-hour equilibration time. Thereafter, one cumulative
dose-response curve (rate and contractile force) for one MDI analogue was obtained. Sustained peak effect for a given concentration of MDI in the range of $10^{-8}$ M to $3 \times 10^{-4}$ M, was observed within 2 to 3 minutes of addition to the organ bath, with the exception of the highest concentrations of tertiary MDIs ($10^{-4}$ M and $3 \times 10^{-4}$ M), particularly the bu-MDI, which required 5 to 10 minutes to display sustained peak effects in this preparation. The mean time courses for generation of the entire dose-response curve for each of the MDI analogues were (minutes, mean ± SEM): bu-MDI ($30.9 \pm 2.4$), Q-bu-MDI ($32.7 \pm 1.2$) and pr-MDI ($32.0 \pm 3.9$).

Reversibility of the negative inotropic effect of pr-MDI

Electrically stimulated guinea pig left atria (prepared, monitored and stimulated as described in the preceding section, using a Krebs-Henseleit solution of identical composition) were allowed an equilibration period of 60 minutes in normal Krebs-Henseleit medium, with changes in bathing solution every 5 minutes. Following equilibration, pr-MDI was added cumulatively to achieve concentrations of $10^{-6}$ M, $10^{-5}$ M and $10^{-4}$ M. Following peak reduction in atrial contractile force, reversal of the pr-MDI-mediated negative inotropic effect was attempted (in the continued presence of the MDI) via: (a) the introduction of an additional 2.5 mM Ca++, thereby increasing the 2.5 mM calcium content of the medium to 5.0 mM; (b) the cumulative
addition of isoproterenol, achieving concentrations of \(10^{-6}\) M, \(10^{-8}\) M and \(10^{-7}\) M; or (c) the cumulative addition of ouabain, achieving concentrations of \(10^{-7}\) M and \(10^{-6}\) M. Contractile responses were expressed as a percent from the basal force recorded at the end of the equilibration period prior to the introduction of pr-MDI.

Effects of pr-MDI upon concentration-response relationships mediated by positive inotropic agents: Evaluation of membrane channel blocking activity

Two types of physiological bathing solutions were used in this study. For those experiments in which concentration-response curves to isoproterenol or ouabain were performed, a normal (2.5 mM calcium) Krebs-Henseleit solution was used, its composition (g/l) being: NaCl 6.92, KCl 0.35, MgSO\(_4\)\(\cdot\)7H\(_2\)O 0.29, CaCl\(_2\)\(\cdot\)2H\(_2\)O 0.37, KH\(_2\)PO\(_4\) 0.16, NaHCO\(_3\) 2.1, dextrose 2.1. For experiments in which concentration-response curves to calcium were performed, the CaCl\(_2\)\(\cdot\)2H\(_2\)O content (g/l) of the Krebs-Henseleit solution was reduced to 0.074, thereby reducing the calcium composition of the fluid from 2.5 mM to 0.5 mM. The latter medium is henceforth referred to as low calcium Krebs-Henseleit medium. In either solution, isolated guinea pig left atrial preparations were electrically stimulated by square wave impulses of 3 msec duration at a frequency of 3 Hz with twice the threshold voltage, delivered by a Grass S9 stimulator (Grass Instrument Company, Quincy, Mass.) Atrial contractile force was recorded isometrically under 500 mg
preload using a Narco Biosystems force transducer (E and M Instrument Company, Houston, Texas) connected to a Physiograph DMP-4B recorder (E and M Instrument Company).

The effect of pr-MDI upon the calcium-mediated concentration-response (force) relationship in isolated atria was evaluated as described below. Following a 60-minute equilibration period in low calcium Krebs-Henseleit medium, with changes in bathing solution every 5 minutes, an initial control concentration-response curve to calcium was performed on electrically-driven left atrial preparations by the cumulative addition of CaCl$_2$·2H$_2$O until the development of peak tension was achieved. This was followed by a 40-minute washout period with low calcium medium, with change of bathing fluid every 5 minutes. Following washout, the electrically-driven left atrial preparations were incubated with pr-MDI (10$^{-6}$M, 10$^{-5}$M, 10$^{-4}$M or 3 x 10$^{-4}$M) for a period of 20 minutes, followed by the performance of a second cumulative concentration-response curve to calcium in the continued presence of the MDI. Several control preparations (n=5, with no MDI treatment between successive concentration-response curves to calcium) were employed in order to determine and correct for shifts between successive concentration-response curves to this cation. Contractile responses were expressed as a percent of the maximum response to calcium obtained on the initial control concentration-response curve to this cation prior to
exposure to pr-MDI. All values were corrected for the basal contractile force recorded in 0.5 mM calcium (low calcium Krebs-Henseleit medium), and for baseline shifts caused by the intrinsic negative inotropic action of pr-MDI.

The protocol for the evaluation of the effect of pr-MDI upon the isoproterenol-mediated concentration-response relationship in isolated guinea pig left atria was generally similar to that described immediately above. Following a 60-minute equilibration period in normal Krebs-Henseleit medium, with changes in bathing fluid every 5 minutes, an initial control concentration-response to isoproterenol was performed on the electrically-driven left atrial preparation until the development of peak tension was achieved. This was followed by a 40-minute washout period, with change of bathing fluid every 5 minutes. Subsequently, the electrically-driven atrial preparations were incubated with pr-MDI ($10^{-6}$M, $10^{-5}$M, $10^{-4}$M or $3 \times 10^{-4}$M) for a period of 20 minutes, followed by the performance of a second cumulative concentration-response curve to isoproterenol in the continued presence of pr-MDI. Several control preparations (n=6, with no MDI treatment between successive isoproterenol curves) were employed in order to determine and correct for shifts between successive isoproterenol concentration-response curves. Contractile response was expressed as a percent of the maximum response to isoproterenol obtained on the initial control
concentration-response curve to this beta-adrenergic agonist performed in the absence of pr-MDI. All values were corrected for the basal contractile force recorded during equilibration in normal Krebs-Henseleit medium prior to the introduction of any drugs, and for baseline shifts caused by the intrinsic negative inotropic action of pr-MDI.

The protocol for the evaluation of the effect of pr-MDI upon the concentration-response relationship mediated by ouabain in isolated guinea pig left atria differs slightly from the two described immediately above. Due to the toxic effects of higher concentrations of ouabain on the tissues, only one cumulative concentration-response curve to ouabain was performed on each preparation. Following a 60-minute equilibration period in normal Krebs-Henseleit medium, with changes in bathing fluid every 5 minutes, the contractile response of each electrically-driven left atrial preparation to a challenge of $10^{-7}$M isoproterenol was determined. This concentration of isoproterenol produces a maximum control contractile response which was used for subsequent calculations (see below). The isoproterenol challenge was followed by a 40-minute washout period, with changes in bathing fluid every 5 minutes. Following washout, the preparations were incubated with pr-MDI ($10^{-6}$M, $10^{-5}$M, $10^{-4}$M or $3 \times 10^{-4}$M) for 20 minutes, followed by the performance of a cumulative concentration-response curve to ouabain. Contractile response
toward ouabain was expressed as a percent of the maximum response obtained with the $10^{-7}$M isoproterenol challenge in the absence of pr-MDI. All values were corrected for the basal contractile force recorded during equilibration in normal Krebs-Henseleit medium prior to the introduction of any drugs, and for baseline shifts caused by the intrinsic negative inotropic action of pr-MDI.

Comparison of the characteristics of the negative inotropic actions of pr-MDI and inhibitors of mitochondrial energy production

English short-haired guinea pigs (Carr Animal Supply, Powell, Ohio) of either sex, weighing 500-1000 g were killed by a sharp blow on the head. The left atria were quickly removed by dissection and suspended in physiological salt solution bubbled with 95% $O_2$ and 5% $CO_2$ at 32°C. The normal (2.5 mM calcium) physiological Krebs-Henseleit solution consisted of (g/l): NaCl 6.92, KCl 0.35, MgSO$_4$·7H$_2$O 0.29, CaCl$_2$·2H$_2$O 0.37, KH$_2$PO$_4$ 0.16, NaHCO$_3$ 2.1, dextrose 2.1. The atrial preparations were electrically stimulated by square wave impulses of 3 msec duration at a frequency of 3 Hz with twice the threshold voltage (determined at the midpoint of a 60-min equilibration period), delivered by a Grass S9 stimulator (Grass Instrument Co., Quincy, Mass.). Atrial contractile force was recorded isometrically under 500 mg preload using Grass FT03C force-displacement transducers connected to a Grass Model 7D
polygraph. The electrically stimulated atrial preparations were allowed an equilibration period of 60 mins in normal Krebs-Henseleit medium, with frequent changes in bathing solution. Following equilibration, one of the cardio-depressants (pr-MDI, dinitrophenol, rotenone, or antimycin A) was added cumulatively to achieve a negative inotropic effect amounting to roughly a 50% (range = 40-55%) reduction in force of contraction with each agent. After a stable reduction in force was achieved, reversal of the negative inotropic effect of each cardiodepressant was attempted (in the continued presence of the cardiodepressant) by the introduction to the bath of either an additional 2.5 mM calcium (thereby increasing the medium calcium concentration to 5 mM), or isoproterenol (added cumulatively to achieve concentrations of 10^{-9} M, 10^{-8} M, and 10^{-7} M). The cardiac stimulants were left in contact with the tissue for 30 mins and their actions monitored. Contractile responses were expressed as percent changes from the basal intrinsic force of contraction (designated as 0% in the figures for this section) recorded at the end of the equilibration period prior to the introduction of any drugs.

**Statistical analysis for isolated atrial studies**

Values are presented as mean±S.E.M. unless otherwise indicated. A paired Student's t-test was used to compare predrug and postdrug threshold voltages for each
left atrial preparation, as well as for comparing developed tension at each frequency in the frequency-force profile of each left atrial preparation in the evaluation of the effects of the MDIs upon atrial mechanical and electrical activity. Statistical analysis of the effects of pr-MDI on contractile responses (after correcting for the intrinsic negative inotropic effects of pr-MDI) mediated by positive inotropic agents was done on a dose-by-dose basis for each positive inotropic using an analysis of variance for a one-way randomized design. Statistically different F values were then tested using a paired Student's t-test for those experiments in which the responses of MDI-treated tissues could be compared to their own paired controls, or by Duncan's New Multiple-Range test. In the comparison of reversibilities of cardiodepressions induced by pr-MDI and by inhibitors of mitochondrial energy production, statistical comparisons of contractile responses were done using an analysis of variance for a one-way randomized design. Statistically different F-values were then tested using the Dunnett test for multiple comparisons. Non-repeated single comparisons of selected treatment responses were done using the Student t-test (Winer, 1971). The positive inotropic action of either cardiostimulant in presence of a cardiodepressant represents the total response of the cardiostimulant measured from the point of peak reduction in force of contraction induced by the cardiodepressant.
In all cases, the level of significance was set at \( p < 0.05 \).

**Intracellular Localization of Pr-MDI in the Isolated, Perfused Guinea Pig Heart**

**Synthesis of \(^{14}\)C-pr-MDI**

The \(^{14}\)C label was introduced in the 3-position of the indene ring, using a slight modification of the original procedure for the synthesis of nonlabeled pr-MDI (Witiak et al., 1974), adapted for the micromolar scale synthesis of \(^{14}\)C-pr-MDI. \(^{14}\)C-labeled dimethylformamide (DMF, 22 \( \mu l \); 0.28 mmol, 54 mCi/mmol) was allowed to warm to room temperature. POCl\(_3\) (14 \( \mu l \); 0.15 mmol) was added to the DMF and the solution stirred for 30 mins at room temperature. To this solution was added 20 \( \mu l \) (0.095 mmol) of the olefin (E)-1-(3,4-methylenedioxyphenyl) pent-1-ene, and the stirred reaction mixture was heated at 89°C for 2 hrs in a sand bath. The resultant dark reaction mixture was quenched by the addition of 2 ml of ice-cold water, and the unreacted olefin was extracted into three 2 ml aliquots of ether, while the hydrochloride salt of the MDI remained in the aqueous layer. The aqueous layer was made basic by the dropwise addition of 10\% NaOH until the solution became cloudy. The basic solution was extracted with five 2 ml aliquots of ether which extracted the MDI base into the organic phase. The combined ether phases containing the MDI were dried with Na\(_2\)SO\(_4\) then acidified with HCl gas.
Some crystals of pr-MDI formed while standing in the freezer; however the mother liquor still contained over 90% of the MDI as visualized by UV and by a Baird Beta Camera. The $^{14}$C-pr-MDI hydrochloride product appeared to be greater than 95% radiochemically pure by TLC; however absolute quantification of purity was difficult due to the slight tailing of the compound (silica GF eluted with isopropanol: acetonitrile, 1:1). The specific activity of $^{14}$C-pr-MDI hydrochloride was 52 mCi/mmol.

Cardiac effects in the isolated, perfused guinea pig heart

The time course of pr-MDI effects on cardiac inotropy and chronotropy was investigated in short-haired guinea pigs of either sex (Carr Animal Supply, Powell, OH), weighing 600-1000 g. The animals were killed by a blow on the head, and the hearts were rapidly excised and prepared for Langendorff perfusion. A peristaltic perfusion pump (Model 375A, 2.5 mm tubing diameter; Sage Instruments, Cambridge, Mass.) delivered the perfusion fluid retrogradely into the coronary vasculature through the aortic stump at a constant rate of 6 ml/min. A 3-way stopcock allowed instantaneous changes in perfusion fluid. The composition of the physiological Krebs-Henseleit solution was (g/l): NaCl 6.896, KCl 0.358, CaCl$_2$·2H$_2$O 0.367, NaHCO$_3$ 2.285, MgSO$_4$·7H$_2$O 0.296, KH$_2$PO$_4$ 0.136, and dextrose 2.0. The MDI was also dissolved in Krebs-Henseleit medium. All perfusion fluids were aerated with 95% $O_2$/5% CO$_2$ and delivered
at 37°C. Heart rate and isometric force of contraction were recorded with Narco Biosystems force transducers (E and M Instruments Co., Houston, Texas) connected to a Physiograph DMP-4B recorder (E and M Instrument Co.). A resting diastolic tension of 2 g was applied to each heart. The hearts were equilibrated for 15-20 mins by perfusion with Krebs-Henseleit solution in order to establish baseline force and rate of contraction. Subsequently, the inotropic and chronic responses of the hearts were monitored for up to 45 mins during perfusion with $3 \times 10^{-5}$M pr-MDI (with or without tracer amounts of $^{14}$C-pr-MDI). Control hearts were perfused for equivalent periods of time with drug-free physiological solution.

**Tissue uptake and washout**

Determination of the washout characteristics of $^{14}$C-pr-MDI in the isolated perfused guinea pig heart was made by measurement of the effluent content of $^{14}$C during the course of the 45 min perfusion with $3 \times 10^{-5}$M pr-MDI (with a mean 0.13% ± 0.03% of drug present as $^{14}$C-pr-MDI tracer) and the subsequent 45 min washout with drug-free Krebs-Henseleit solution. The effluent samples were collected at 1, 2, 4, 8, 16, 30 and 45 mins of drug perfusion, and at 1, 2, 4, 8, 16, 30 and 44 mins of drug-free washout. Aliquots of effluent were added to Thrift Solve Scintillation solution (Kew Scientific, Columbus, OH), and were counted in a Beckman LS-6800 liquid scintillation
counter (Beckman Instruments, Palo Alto, California) using H-number to monitor and correct for quench. Counting efficiency was >90%. From the DPMs of $^{14}$C in the effluent samples, the total effluent concentration of pr-MDI was calculated.

**Subcellular fractionation of guinea pig ventricles**

Guinea pig ventricles were excised from hearts perfused with drug-containing Krebs-Henseleit solution and from non-perfused hearts and analyzed separately. The individual ventricles were placed in ice-cold solution containing sucrose (250 mM), Tris HCl (10 mM) and EDTA (2 mM), pH 7.4, at 4°C. All subsequent separation steps were carried out at 4°C. The ventricles were minced and homogenized in a Polytron tissue homogenizer (Brinkman Instruments, Westbury, N.Y.) at low speed for 5 sec, followed by three 5 sec periods at maximum speed. The resulting homogenate was passed three times through an all glass homogenizer, and then filtered through surgical gauze. The filtered homogenate was then diluted with sucrose-Tris-EDTA buffer (4°C) to an approximately 25% suspension (8-9 ml of whole homogenate diluted to a final volume of 35 ml). The diluted homogenate was centrifuged at 1085 x g (avg.) for 15 mins (repeated once for 10 mins) in a Sorvall RC-5B centrifuge (Dupont Industries, Newton, Conn.). The pellet from the 1085 x g centrifugations (designated the nuclear/cell debris fraction) was resuspended in 3-4 ml of sucrose-Tris-EDTA
buffer in an all glass homogenizer. The combined 1085 x g supernatants were centrifuged at 14,500 x g (avg.) for 20 mins (repeated once). The 14,500 x g pellet (designated the mitochondrial fraction) was resuspended in 4-5 ml sucrose-Tris-EDTA buffer in an all glass homogenizer. The combined 14,500 x g supernatants were centrifuged at 109,000 x g (avg.) in a Beckman L5-75 preparative ultra-centrifuge for 60 mins. The 109,000 x g supernatant (designated as the supernatant or cytosolic fraction) was separated from the pellet. The 109,000 x g pellet was resuspended in approximately 2 ml of sucrose-Tris-EDTA buffer in a teflon-glass homogenizer, and was layered onto a discontinuous sucrose density gradient made up of (bottom to top) 4 ml of 45% sucrose, 3 ml of 33% sucrose, and 3 ml of 28% sucrose, as described by Wei et al. (1976). The gradient was centrifuged at 112,000 x g (avg.) for 120 mins in a swinging bucket SW-36 rotor (Beckman Industries). Protein bands were then harvested from the three interfaces using Pasteur pipettes, and the resultant three fractions were diluted with Tris HCl (10 mM)-EDTA (2 mM) buffer and centrifuged at 122,000 x g (avg.) for 60 mins to yield three pellets. Each 122,000 x g pellet was resuspended in 1.0-1.5 ml of sucrose-Tris-EDTA buffer in a teflon-glass homogenizer, and these final three fractions were designated as interfaces 1, 2, and 3 corresponding to the top, middle, and lower protein bands of the density gradient, respectively.
Characterization of subcellular fractions

The relative purity of each subcellular fraction was determined by assays of Marker enzymes. Protein content in each subcellular fraction was determined by the method of Lowry et al. (1951). A Gilford model 250 spectrophotometer attached to a Gilford model 6050 recorder (Gilford Instruments, Oberlin, Ohio) was utilized in the measurement of enzyme activities and protein concentrations. ATPase activities were determined by a modification of the method of Kidwai et al. (1971). Total ATPase activity (Na\(^+\) + Mg\(^{2+}\) + K\(^+\)) was determined in a reaction mixture of 1 ml containing 10 mM Tris HCl (pH 7.4 at 25°C) with 50 mM histidine, 5 mM MgCl\(_2\), 100 mM NaCl, 10 mM KCl, 25-100 \(\mu\)g of fraction protein, and 3 mM ATP (Mg salt). The reaction was started by adding the ATP, and the mixture was incubated at 37°C for 10 mins. The reaction was stopped by adding 1 ml of 12% trichloroacetic acid. After centrifugation at 12,000 \(\times\) g (avg.), the liberated inorganic phosphate was determined by the method of Fiske and Subbarow (1925). That fraction of total ATPase activity designated as Na\(^+\)/K\(^+\)-ATPase activity was calculated as the difference between activities in the presence and in the absence of 1 mM ouabain in the reaction mixture. NADPH cytochrome c-reductase activity was measured by the spectrophotometric method of Phillips and Langdon (1962) in 25-200 \(\mu\)g of
fraction protein. Succinate cytochrome c-reductase activity was determined by the spectrophotometric method of Tisdale (1967) in 2 μg of fraction protein.

**Tissue concentration and subcellular distribution**

The determination of pr-MDI uptake into cardiac atrial and ventricular tissues, and its intracellular localization in ventricular cells, was made by measurement of the content of $^{14}$C-pr-MDI tracer in tissue samples, subcellular fractions, and effluent aliquots from isolated guinea pig hearts which had been perfused with $3 \times 10^{-5}$M pr-MDI (with a mean $0.37\% \pm 0.03\%$ of drug present as $^{14}$C-pr-MDI tracer) for 8, 15, 30, or 45 mins. Subsequent to drug perfusion, the hearts were immediately perfused with drug-free Krebs-Henseleit medium for a 10-min washout period. Tissue samples were then taken from the right and left atria and ventricles (15-50 mg), and the remainder of the ventricles were homogenized and subjected to differential and density gradient centrifugation as described above to obtain the various subcellular fractions. The atrial and ventricular whole tissue samples and aliquots of the ventricular subcellular fractions were incubated in Protosol (New England Nuclear, Boston, Mass.) for 24 hrs at 50°C for protein solubilization, and then neutralized with glacial acetic acid. The digested samples were then added to Thrift-Solv emulsion-type scintillation solution, and
counted in a Beckman LS-6800 liquid scintillation counter using H-number to monitor and correct for quench. From the DPMs of $^{14}$C in each sample, the total content of pr-MDI was calculated for whole tissue and for each subcellular fraction. Effluent samples from the perfused hearts were also counted for $^{14}$C, as described above, and the data obtained used for calculating the tissue/medium ratios for each time interval of drug exposure.

**Analysis of data from isolated, perfused heart studies**

Values are presented as mean±S.E.M. unless otherwise indicated. Kinetic analysis of pr-MDI tissue washout data was done by fitting MDI effluent concentrations to both a monoexponential and a biexponential model with the aid of the NONLIN computer program (Metzler et al., 1974). Nonrepeated single comparisons of selected enzyme activities or drug concentrations were done using paired or unpaired Student t-tests (Winer, 1971). In all cases, the level of significance was set at $p<0.05$. 
CHAPTER III

RESULTS

Evaluation of MDIs in In Vivo Models of Arrhythmia

Calcium-induced arrhythmias in dogs

In control dogs, the mean time to onset of arrhythmias was 66.8 ± 4.4 minutes and the mean time to death was 77.6 ± 5 minutes from the time of initiating the calcium infusion (Table 1). The majority of the control dogs exhibited an initial tachycardia followed by bradycardia prior to the onset of arrhythmias. Typical alterations in the ECGs of control animals prior to the onset of arrhythmias included fluctuations in height and form of P and T waves, depression of the ST segment, and reduction in QRS amplitude. The predominant types of calcium-induced arrhythmias in these animals were coupled ectopic beats, sino-atrial block, atrio-ventricular dissociation with or without nodal rhythm and ventricular fibrillation.

Pretreatment of dogs with pr-MDI or bu-MDI resulted in a significant delay (p < 0.05) in both the onset of calcium-induced arrhythmias and the time to death (Table 1), with the pr-MDI appearing somewhat more effective than the bu-MDI. In addition, the pr-MDI virtually abolished the
TABLE 1. The effects of MDI pretreatment on calcium-induced arrhythmias in dogs

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Time to onset of arrhythmias (min)</th>
<th>Time to death&lt;sup&gt;a&lt;/sup&gt; (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 10)</td>
<td>66.8 ± 4.4</td>
<td>77.6 ± 5.0</td>
</tr>
<tr>
<td>Pr-MDI 30 mg/kg (n = 5)</td>
<td>92.0 ± 10.6</td>
<td>121.3 ± 14.4</td>
</tr>
<tr>
<td>Bu-MDI 30 mg/kg (n = 6)</td>
<td>78.0 ± 3.8</td>
<td>101.0 ± 7.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>All dogs died either in ventricular fibrillation or in cardiac arrest (total SA arrest or total AV block followed by SA arrest).
incidence of the initial calcium-induced tachycardia while the bu-MDI only diminished its magnitude and duration. The general types of calcium-induced ECG changes and arrhythmias which eventually developed after the delayed onset in the MDI-pretreated animals were similar to those observed in the control dogs.

**Calcium-induced arrhythmias in rats**

In control rats, the intravenous administration of an acute dose of calcium chloride elicited arrhythmias which are characterized in Table 2. Within 30 seconds of calcium administration a profound bradycardia was evident. Complex arrhythmias, predominantly composed of sino-atrial block, 2nd and 3rd degree atrio-ventricular block, ectopic beats (nodal and ventricular), and ventricular flutter and fibrillation occurred in 95% of these animals during and after the initial bradycardia, ending in death of 30% of the controls. The mean time for reversion to control ECG in surviving animals was $332 \pm 57$ seconds. (Since the vehicles had no demonstrable pharmacological properties, all the randomly run controls were pooled and appear in Tables 2-6).

Pretreatment of rats with 3.75 mg/kg of either the pr- or bu-MDI (Table 2) afforded virtually complete protection against calcium-induced arrhythmias and mortality, and prevented the occurrence of the initial phase of calcium-induced bradycardia. However, only the bu-MDI at
Table 2. The effects of pr- and bu-MDI pretreatments on cardiac function and calcium-induced arrhythmias in rats.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Immediate AHR due to pretreatment*1 (%)</th>
<th>AHR 10 mins after pretreatment*1 (%)</th>
<th>ΔECG due to pretreatment*1,2,3</th>
<th>ΔHR due to calcium*4 (%)</th>
<th>Incidence of calcium-induced arrhythmias</th>
<th>Reversion time (secs)</th>
<th>Death*5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>41.8±0.6</td>
<td>42.9±0.7</td>
<td>0/20</td>
<td>432.2±7.3</td>
<td>16/20 16/20 11/20 13/20 19/20 6/20</td>
<td>332±57</td>
<td>6/20</td>
</tr>
<tr>
<td>Pr-MDI 2.5 ng/kg</td>
<td>43.5±0.9</td>
<td>44.3±1.1</td>
<td>0/10</td>
<td>424.1±9.5</td>
<td>6/10 6/10 4/10 3/10 7/10 4/10</td>
<td>199±35</td>
<td>2/10</td>
</tr>
<tr>
<td>Pr-MDI 3.75 ng/kg</td>
<td>44.2±1.2</td>
<td>45.2±1.4</td>
<td>0/10</td>
<td>4.2±2.2</td>
<td>4/10 4/10 0/10 4/10 4/10 0/10</td>
<td>255±53</td>
<td>0/10</td>
</tr>
<tr>
<td>Bu-MDI 2.5 ng/kg</td>
<td>41.2±0.5</td>
<td>41.8±1.0</td>
<td>0/10</td>
<td>422.0±7.3</td>
<td>4/10 5/10 4/10 4/10 7/10 4/10</td>
<td>252±43</td>
<td>0/10</td>
</tr>
<tr>
<td>Bu-MDI 3.75 ng/kg</td>
<td>44.4±0.6</td>
<td>44.4±1.2</td>
<td>0/10</td>
<td>2.2±2.2</td>
<td>0/10 0/10 1/10 0/10 1/10 0/10</td>
<td>148±30</td>
<td>0/10</td>
</tr>
</tbody>
</table>

*1 Heart rate (HR) expressed as percent change compared to pretreatment HR.
*2 Number of animals.
*3 Changes in wave form or amplitude.
*4 Expressed as percent change in heart rate compared to the rate immediately before calcium administration.
*5 p < 0.05 as compared to control value.
this dose induced a significant reduction (p < 0.05) in the time for reversion to control ECG. Pretreatment of rats with 2.5 mg/kg of either MDIs (Table 2) provided only marginal protection against calcium-induced toxicity, although the pr-MDI at this dose significantly reduced the time for reversion to normal ECG in surviving animals (p < 0.05) while the bu-MDI fully protected against calcium-induced mortality. It is noteworthy that the MDIs (and the control vehicle) had no intrinsic effects on heart rate or ECG (Table 2).

Interestingly, the Q-bu-MDI, which was synthesized primarily for comparative purposes (the tertiary pr- and bu-MDIs having a putative intracellular site of action; and the charged Q-bu-MDI, being less likely to cross biological membranes, would be confined to extracellular space), was clearly more potent than its tertiary analogues, the optimum protective dose of the quaternary compound being 0.5 mg/kg. At this dose, Q-bu-MDI afforded complete protection against arrhythmias and mortality induced by calcium, and significantly reduced the time for reversion to normal ECG. At the doses tested (0.25-1 mg/kg), Q-bu-MDI did not exhibit any intrinsic activity on the heart during the pretreatment period (Table 3).

The protective activities of the MDI analogues, particularly that of the Q-bu-MDI, in this evaluation compared very favorably to the protective activities of several standard antiarrhythmics. The pretreatment of rats with
Table 3. The effects of Q-bu-MDI and quinidine pretreatments on cardiac function and calcium-induced arrhythmias in rats.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Immediate ΔHR due to pretreatment (%)</th>
<th>ΔHR 10 mins after pretreatment (%)</th>
<th>ΔECC due to pretreatment a,b</th>
<th>ΔHR due to calcium c (%)</th>
<th>Incidence of calcium-induced arrhythmias a</th>
<th>Reversion time (secs)</th>
<th>Death d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control a, (n=20)</td>
<td>+ 1.8 ± 0.6</td>
<td>+ 2.9 ± 0.7</td>
<td>0/20</td>
<td>+ 32.2 ± 7.3</td>
<td>16/20</td>
<td>16/20</td>
<td>11/20</td>
</tr>
<tr>
<td>Q-bu-MDI 0.25 mg/kg (n=10)</td>
<td>+ 1.7 ± 1.0</td>
<td>+ 4.2 ± 1.2</td>
<td>0/10</td>
<td>+ 13.7 ± 3.4 d</td>
<td>3/10</td>
<td>3/10</td>
<td>4/10</td>
</tr>
<tr>
<td>0.5 mg/kg (n=10)</td>
<td>+ 3.7 ± 1.2</td>
<td>+ 0.8 ± 1.2</td>
<td>0/10</td>
<td>+ 10.4 ± 2.3 d</td>
<td>1/10</td>
<td>0/10</td>
<td>3/10</td>
</tr>
<tr>
<td>1.0 mg/kg (n=10)</td>
<td>+ 2.4 ± 1.1</td>
<td>+ 2.8 ± 1.2</td>
<td>5/10</td>
<td>+ 12.9 ± 3.2 d</td>
<td>1/10</td>
<td>2/10</td>
<td>5/10</td>
</tr>
<tr>
<td>Quinidine 4.0 mg/kg (n=10)</td>
<td>+ 4.6 ± 0.8</td>
<td>+ 2.8 ± 1.0</td>
<td>10/10</td>
<td>+ 15.6 ± 2.8</td>
<td>4/10</td>
<td>3/10</td>
<td>6/10</td>
</tr>
<tr>
<td>8.0 mg/kg (n=10)</td>
<td>+ 5.4 ± 1.7</td>
<td>+ 0.9 ± 1.4</td>
<td>10/10</td>
<td>+ 19.1 ± 2.8</td>
<td>2/10</td>
<td>1/10</td>
<td>2/10</td>
</tr>
</tbody>
</table>

NOTE: The rats were pretreated intravenously with quinidine, Q-bu-MDI, or 5% dextrose 10 min prior to intravenous administration of calcium.

a Number of animals.
b Refer to text for ECG alterations.
c Expressed as percent change in heart rate compared to the rate immediately before calcium administration.
d P < 0.05 as compared to control value. Statistical analysis performed on actual rate changes (beats/min).
e P < 0.05 as compared to control value.
2.0, 3.0, or 3.75 mg/kg of diphenylhydantoin (the mechanism of action of which may involve inhibition of transmembrane calcium influx) afforded marginal to no protection against calcium-induced arrhythmias or mortality, and failed to prevent the initial calcium-induced bradycardia or to reduce the time to reversion to normal ECG in surviving animals. Doses of diphenylhydantoin up to 3.75 mg/kg (and the control vehicle) exhibited no intrinsic effects on heart rate or ECG. The 5.0 mg/kg dose resulted in changes in wave shape and amplitude in the ECG, and afforded protection only against calcium-induced bradycardia (Table 4).

Pretreatment of rats with 2.0, 3.0 or 3.75 mg/kg of prenylamine, a calcium entry blocker, (Table 5) afforded marginal to no protection against calcium-induced arrhythmias or mortality, and failed to prevent the initial calcium-induced bradycardia or to reduce the time to reversion to normal ECG in surviving animals. Prenylamine exhibited intrinsic properties of its own, and pretreatment with this drug resulted in bradycardia (at 3.0 and 3.75 mg/kg doses) and ECG changes ranging from fluctuations in wave form and amplitude (at 2.0, 3.0 and 3.75 mg/kg doses) to atrioventricular and sino-atrial block (at the 3.75 mg/kg dose).

Pretreatment of rats with verapamil, a calcium entry blocker, produced dose-dependent effects on calcium-induced toxicity as depicted in Table 6. A dose of 1.0 mg/kg of verapamil was virtually without effect on the
Table 4. The effects of diphenylhydantoin (DPH) pretreatment on cardiac function and calcium-induced arrhythmias in rats.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Immediate ΔHR due to pretreatment a (%)</th>
<th>ΔHR 10 mins after pretreatment a (%)</th>
<th>ΔECG due to pretreatment b,c</th>
<th>ΔHR due to calcium d (%)</th>
<th>Incidence of calcium-induced arrhythmias</th>
<th>Reversion time (secs)</th>
<th>Death b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 20)</td>
<td>41.8±0.6</td>
<td>42.9±0.7</td>
<td>0/20</td>
<td>432.2±7.3</td>
<td>16/20 16/20 11/20 13/20 19/20</td>
<td>332±57</td>
<td>6/20</td>
</tr>
<tr>
<td>DPH 2.0 mg/kg (n = 10)</td>
<td>43.5±0.5</td>
<td>45.5±1.6</td>
<td>0/10</td>
<td>426.1±8.2</td>
<td>6/10 6/10 7/10 7/10 10/10</td>
<td>246±24</td>
<td>3/10</td>
</tr>
<tr>
<td>DPH 3.0 mg/kg (n = 10)</td>
<td>42.6±1.0</td>
<td>44.1±1.1</td>
<td>0/10</td>
<td>427.4±7.6</td>
<td>8/10 5/10 2/10 6/10 10/10</td>
<td>259±61</td>
<td>2/10</td>
</tr>
<tr>
<td>DPH 3.75 mg/kg (n = 10)</td>
<td>45.2±0.7</td>
<td>47.1±1.3</td>
<td>0/10</td>
<td>432.2±11.7</td>
<td>7/10 4/10 5/10 7/10 8/10</td>
<td>284±51</td>
<td>2/10</td>
</tr>
<tr>
<td>DPH 5.0 mg/kg (n = 10)</td>
<td>45.8±0.6</td>
<td>49.6±1.1</td>
<td>4/10</td>
<td>412.8±6.8 e</td>
<td>6/10 6/10 6/10 6/10 8/10</td>
<td>339±97</td>
<td>3/10</td>
</tr>
</tbody>
</table>

a Heart rate (HR) expressed as percent change compared to pretreatment HR.
b Number of animals.
c Changes in wave form or amplitude.
d Expressed as percent change in heart rate compared to the rate immediately before calcium administration.
e p < 0.05 as compared to control value.
Table 5. The effects of pirenzepine pretreatment on cardiac function and calcium-induced arrhythmias in rats

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Immediate ΔHR due to pretreatment (%)</th>
<th>ΔHR 10 mins after pretreatment (%)</th>
<th>ΔECG due to pretreatment (%)</th>
<th>ΔHR due to calcium (%)</th>
<th>Incidence of calcium-induced arrhythmias</th>
<th>Reversion time (secs)</th>
<th>Death (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SA block</td>
<td>AV block</td>
<td>Ectopic beats</td>
</tr>
<tr>
<td>Control (n = 20)</td>
<td>+1.8±0.6</td>
<td>+2.9±0.7</td>
<td>0/20</td>
<td>432.2±7.3</td>
<td>16/20</td>
<td>16/20</td>
<td>11/20</td>
</tr>
<tr>
<td>Pirenzepine 2.0 mg/kg (n = 10)</td>
<td>+2.6±0.7</td>
<td>+4.9±0.8</td>
<td>3/10</td>
<td>427.1±4.2</td>
<td>8/10</td>
<td>6/10</td>
<td>4/10</td>
</tr>
<tr>
<td>Pirenzepine 3.0 mg/kg (n = 10)</td>
<td>+10.5±2.2</td>
<td>+25.7±1.0</td>
<td>5/10</td>
<td>417.0±7.1</td>
<td>10/10</td>
<td>7/10</td>
<td>4/10</td>
</tr>
<tr>
<td>Pirenzepine 3.75 mg/kg (n = 10)</td>
<td>+15.1±3.1</td>
<td>+27.5±3.7</td>
<td>8/10c</td>
<td>433.4±10.0</td>
<td>6/10</td>
<td>5/10</td>
<td>5/10</td>
</tr>
</tbody>
</table>

*Heart rate (HR) expressed as percent change compared to pretreatment HR.

bNumber of animals.

Changes in wave form or amplitude.

Expressed as percent change in heart rate compared to the rate immediately before calcium administration.

In addition to changes in ECG wave form and amplitude, one rat exhibited SA block and another exhibited AV block.
Table 6. The effects of verapamil pretreatment on cardiac function and calcium-induced arrhythmias in rats.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Immediate AHR due to pretreatment&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>AHR 10 mins after pretreatment&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>AECG due to pretreatment&lt;sup&gt;b,c&lt;/sup&gt;</th>
<th>Immediate AHR due to calcium&lt;sup&gt;d&lt;/sup&gt; (%)</th>
<th>Incidence of calcium-induced arrhythmias&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Reversion time (secs)</th>
<th>Death&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 20)</td>
<td>1.8±0.6</td>
<td>2.9±0.7</td>
<td>0/20</td>
<td>432.2±7.3</td>
<td>16/20 16/20 11/20 13/20 19/20</td>
<td>332±57</td>
<td>6/20</td>
</tr>
<tr>
<td>Verapamil 1.0 mg/kg</td>
<td>425.3±2.0</td>
<td>413.3±1.4</td>
<td>8/10</td>
<td>418.8±5.0</td>
<td>4/10 6/10 7/10 5/10 8/10</td>
<td>233±19</td>
<td>2/10</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verapamil 2.0 mg/kg</td>
<td>429.6±1.7</td>
<td>418.0±1.4</td>
<td>9/10</td>
<td>417.4±7.4</td>
<td>4/10 3/10 6/10 2/10 6/10</td>
<td>252±42</td>
<td>2/10</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Verapamil 3.0 mg/kg</td>
<td>434.7±3.5</td>
<td>423.5±2.8</td>
<td>10/10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>416.1±4.8&lt;sup&gt;g&lt;/sup&gt;</td>
<td>3/10 3/10 4/10 1/10 4/10</td>
<td>154±24&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0/10</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verapamil 3.75 mg/kg</td>
<td>436.9±3.6</td>
<td>432.3±2.7</td>
<td>10/10&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4.5±2.4&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0/10 0/10 0/10 0/10 0/10</td>
<td>163±31&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0/10</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Heart rate (HR) expressed as percent change compared to pretreatment HR.

<sup>b</sup>Number of animals.

<sup>c</sup>Changes in wave form and amplitude.

<sup>d</sup>Expressed as percent change in heart rate compared to the rate immediately before calcium administration.

<sup>e</sup>In addition to changes in ECG wave form and amplitude, 3 rats exhibited AV-block.

<sup>f</sup>In addition to changes in ECG wave form and amplitude, 7 rats exhibited AV-block.

<sup>g</sup><i>p < 0.05</i> as compared to control value.
calcium-induced parameters measured. The 2.0 mg/kg dose of verapamil provided some protection only against calcium-induced atrioventricular block and ventricular fibrillation. The 3.0 mg/kg dose of verapamil afforded clear protection against calcium-induced arrhythmias, completely prevented mortality, and significantly reduced the time for reversion to control ECG ($p < 0.05$). The 3.75 mg/kg dose of verapamil afforded complete protection against calcium-induced bradycardia, and significantly reduced the time for reversion to control ECG ($p < 0.05$). Verapamil exhibited intrinsic properties of its own, and pretreatment with all doses of this drug resulted in immediate and marked dose-dependent reduction in heart rate which recovered slightly within 10 minutes with the lower doses. Furthermore, all doses of verapamil resulted in ECG alterations in wave form and amplitude, while the higher doses (3.0 and 3.75 mg/kg) produced a transient atrio-ventricular block.

As shown in Table 3, pretreatment of rats with 4 mg/kg of quinidine, a prototype Class I antiarrhythmic (transmembrane sodium influx inhibitor), afforded partial protection against calcium-induced bradycardia, arrhythmias (except for ectopic beats), and mortality, and significantly reduced the time to reversion to control ECG. Pretreatment with 8 mg/kg of quinidine afforded partial protection against calcium-induced bradycardia, almost complete protection against arrhythmias, and complete protection
against calcium-induced mortality, and significantly re-
duced the time to reversion to control ECG. Both the 4 and
8 mg/kg doses of quinidine, however, produced moderate to
severe alterations in the ECG wave form during pretreatment.

**Aconitine-induced arrhythmias in rats**

Pr-MDI (24 mg/kg), Q-bu-MDI (1 mg/kg) and quinidine
(8 mg/kg) displayed significant protective activity against
aconitine-induced arrhythmias (Figure 2). Other doses of
these agents and the tested doses of bu-MDI displayed only
suggestive antiarrhythmic activity. At the protective
doses, quinidine produced a very slight bradycardia
(-7.8 ± 0.9%) and severe deepening of the S-wave; pr-MDI
profoundly reduced the heart rate (-28.9 ± 4.4%) and in-
creased the QRS amplitude; while Q-bu-MDI had virtually no
effect on heart rate or the normal ECG. At the higher dose
of 4 mg/kg, Q-bu-MDI resulted in severe ECG alterations and
bradycardia (-43 ± 2.5%), with loss of antiarrhythmic
activity.

**Methacholine-induced arrhythmias in rats**

The MDIs did not exhibit significant antiarrhythmic
activity against methacholine-induced arrhythmias, although
a suggestive trend is evident in Figure 3. Only quinidine
(8 mg/kg) produced a significant protection in this evalu-
ation.
Figure 2. Effects of MDI or quinidine pretreatment on aconitine-induced arrhythmias in the rat. (*p< 0.05, compared to control). Inset: Lead II ECG recording before (A) and after (B) aconitine infusion. Scale bar represents one second.
Fig. 2
Figure 3. Effects of MDI or quinidine pretreatment on methacholine-induced arrhythmias in the rat. (*p < 0.05, compared to control). Inset: Lead II ECG recording before (A) and after (B) methacholine infusion. Scale bar represents one second.
Fig. 3
Chloroform/hypoxia-induced arrhythmias in mice

The MDIs and quinidine afforded at least a 70% protection against chloroform-induced ventricular tachycardia (Table 7), the intraperitoneal antiarrhythmic ED50s being: Q-bu-MDI, 10.5 mg/kg; bu-MDI, 44 mg/kg; pr-MDI, 68 mg/kg; and quinidine, 67 mg/kg. Slope functions and tests for parallelism of antiarrhythmic dose-response curves indicated that the individual MDI treatment regression lines were parallel to that of quinidine (p < 0.05), and the ED50s of bu-MDI and Q-bu-MDI were significantly smaller than that of quinidine (p < 0.05). In addition, the therapeutic indices of the MDIs compared favorably with that of quinidine (Table 7).

Evaluation of the MDIs in Isolated Guinea Pig Atrial Preparations

Effects upon mechanical and electrical activity

Figure 4 depicts the effects of the MDI analogues on contractile force in stimulated left guinea pig atria. In 18 atria stimulated at a frequency of 3 Hz, the tension developed at the end of the equilibration period was 0.68 ± 0.11 gram. The pr- and bu-MDIs caused marked, dose-dependent decreases in left atrial contractile force, while the Q-bu-MDI caused only a relatively slight decrease in contractile force at lower concentrations and an increase in force at higher concentrations.
Table 7. The effects of MDIs and quinidine pretreatments on chloroform-induced arrhythmias in mice

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>$n^a$</th>
<th>$\text{ED}_{50}^b$ (mg/kg, i.p.)</th>
<th>$\text{LD}_{50}^b$ (mg/kg, i.p.)</th>
<th>Therapeutic Index ($\text{LD}<em>{50}^b/\text{ED}</em>{50}^b$)</th>
<th>Antiarrhythmic Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinidine</td>
<td>40</td>
<td>67.0 (53.3-84.2)</td>
<td>225 $^c$ (210-241)</td>
<td>3.1</td>
<td>1.00</td>
</tr>
<tr>
<td>Pr-MDI</td>
<td>40</td>
<td>68.0 (52.3-83.4)</td>
<td>185 $^d$ (171.3-199.8)</td>
<td>2.7</td>
<td>0.99</td>
</tr>
<tr>
<td>Bu-MDI</td>
<td>40</td>
<td>44.0 (34.1-56.7)</td>
<td>185 $^d$ (171.3-199.8)</td>
<td>4.2</td>
<td>1.52</td>
</tr>
<tr>
<td>Q-bu-MDI</td>
<td>40</td>
<td>10.5 (4.0-27.9)</td>
<td>65 (56.8-74.4)</td>
<td>6.2</td>
<td>6.38</td>
</tr>
</tbody>
</table>

NOTE: The mice were pretreated intraperitoneally with quinidine or the appropriate MDI 10 min prior to exposure to chloroform.

$^a$ Number of pretreated mice exposed to chloroform.

$^b$ Mean values and 95% confidence limits.

$^c$ Values from Lawson (1968).

$^d$ Values from Rahwan et al. (1979).
Figure 4. Effects of MDIs on the contractile force of isolated guinea pig left atria stimulated at a frequency of 3 Hz. The values (means ± S.E.M., except where the S.E.M. is too small) are calculated as a percentage of the values measured after an initial equilibration period of one hour. Sustained peak effect for a given concentration of any MDI was observed within 3-5 mins of addition to organ bath.
Fig. 4

LEFT ATRIAL CONTRACTILE FORCE (% OF CONTROL VALUE)

pr-MDI (n = 5)

bu-MDI (n = 7)

Q-bu-MDI (n = 6)

MOLAR CONCENTRATION

10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
Figure 5 depicts the effects of the MDI analogues on contractile force in spontaneously beating guinea pig atria. In 15 right atria, the tension developed at the end of the equilibration period was 0.94 ± 0.10 gram. The pr- and bu-MDIs resulted in a decrease in contractile force, particularly at the higher concentrations, while the Q-bu-MDI displayed minimal effects on contractile force except at the highest concentrations where an increase in force of contraction was observed.

Figure 6 depicts the effects of the MDI analogues on spontaneous right atrial rate. At the end of the equilibration period, the atrial rate in 15 preparations was 142 ± 4 beats per minute. At concentrations of 3 x 10^-5 M or less, the three MDI analogues caused only a very slight reduction in right atrial rate. At higher concentrations, the three analogues further decreased right atrial rate, with the tertiary MDIs being more potent than the quaternary analogue. At these higher concentrations, three of five right atria exposed to pr-MDI ceased to beat rhythmically (hence the reduction in n for right atrial rate and force of contraction at higher concentrations of pr-MDI in Figures 5 and 6).

Figure 7 depicts the effects of the MDI analogues, following cumulative addition up to 3 x 10^-4 M, on the frequency-force relationship in stimulated left atria. At this concentration, both the pr-MDI and bu-MDI significantly
Figure 5. Effects of MDIs on the contractile force of spontaneously-beating, isolated guinea pig right atria. The mean values are calculated as a percentage of the values measured after an initial equilibration period of one hour. The number of experiments is given in parentheses. Error bars (except where too small to be shown) represent S.E.M. for \( n > 2 \), and denote range for \( n = 2 \). Sustained peak effect for a given concentration of any MDI was observed within 2-3 mins, with the exception of the highest concentrations (\( 10^{-4} \)M and \( 3 \times 10^{-4} \)M) of the tertiary MDIs which required 5-10 mins to produce peak effects.
Figure 6. Effects of MDIs on the rate of contraction of spontaneously-beating, isolated guinea pig right atria. The mean values are calculated as a percentage of the values measured after an initial equilibration period of one hour. The number of experiments is given in parentheses. Error bars (except where too small to be shown) represent S.E.M. for $n > 2$, and denote range for $n = 2$. Sustained peak effect for a given concentration of any MDI was observed within 2-3 mins with the exception of the highest concentrations ($10^{-4}$M and $3 \times 10^{-4}$M) of the tertiary MDIs which required 5-10 mins to display peak effects.
Fig. 6
Figure 7. Effects of MDIs on frequency-force relationship of isolated guinea pig left atria: Effect of frequency of stimulation (Hz) on mean isometric tension (gm) in the absence (predrug) and the presence (postdrug) of $3 \times 10^{-4} \text{M}$ of indicated MDI. The number of experiments is given in parentheses. Error bars (except where too small to be shown) represent S.E.M. for $N > 2$, and denote range for $n = 2$. $^* P < 0.05$, $^{**} P < 0.01$ (paired $t$-test). No statistics were performed where $n < 3$. 
Fig. 7
depressed the frequency-force curve for the stimulated atria, and both rendered the atria incapable of following electrical stimulation at a frequency of 5 Hz. In addition, this concentration of the pr-MDI analogue rendered several of the left atrial preparations incapable of following electrical stimulation at frequencies of 3 or 4 Hz. The Q-bu-MDI, at the same concentration, did not alter the frequency-force profile of stimulated left atria, nor did it impair the ability of the atria to follow electrical stimulation in the frequency range of 1-5 Hz.

Table 8 presents the effects of the MDI analogues on the threshold voltage required to drive left atria at a frequency of 3 Hz. At the end of the equilibration period, the threshold for electrical stimulation in 17 left atria stimulated at a frequency of 3 Hz was $1.52 \pm 0.09$ volts. Following cumulative addition up to $3 \times 10^{-4}$M, both the pr-MDI and bu-MDI significantly increased the threshold voltage for the stimulated left atria. The Q-bu-MDI, at the same concentration, did not significantly alter threshold voltage.

Reversibility of the negative inotropic effect of pr-MDI

In 14 electrically-stimulated guinea pig left atrial preparations, the basal tension developed at the end of the equilibration period was $715.7 \pm 141.2$ mg, with a threshold voltage of $1.52 \pm 0.15$ volts. Figure 8 - 10 depict the
Table 8. The effect of MDI treatment on the threshold voltage (TV) of guinea pig left atria stimulated at a frequency of 3 Hz

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Predrug TV b (volt)</th>
<th>Postdrug TV b (volt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pr-MDI (n=5)</td>
<td>1.40 ± 0.09</td>
<td>1.82 ± 0.05 c,e</td>
</tr>
<tr>
<td>Bu-MDI (n=8)</td>
<td>1.69 ± 0.20</td>
<td>1.96 ± 0.23 d,f</td>
</tr>
<tr>
<td>Q-bu-MDI (n=4)</td>
<td>1.35 ± 0.09</td>
<td>1.43 ± 0.12</td>
</tr>
</tbody>
</table>

a Cumulative addition up to final concentration of 3x10^-4 M (see Methods).
b Mean ± S.E.M.
c Significantly different from predrug value at p < 0.05 (paired t-test).
d Significantly different from predrug value at p < 0.01 (paired t-test).
e An increase in postdrug TV was observed in all 5 preparations.
f An increase in postdrug TV was observed in 6 of the 8 preparations, while no change was observed in 2 preparations.
Figure 8. Reversibility of the negative inotropic effect of pr-MDI by excess calcium. Pr-MDI was added cumulatively resulting in a maximum negative inotropic effect at $10^{-4} \text{M}$. Thereupon, the calcium concentration in the medium was raised from 2.5 mM to 5.0 mM by addition of 2.5 mM calcium. The immediate and sustained (after 10 mins) effects of elevated calcium are shown. Bars represent mean ± S.E.M. of 4 experiments.
Fig. 8
Figure 9. Reversibility of the negative inotropic effect of pr-MDI by isoproterenol (ISOP). Pr-MDI was added cumulatively resulting in a maximum negative inotropic effect at $10^{-4}$M. Thereupon, isoproterenol was added cumulatively to a final concentration of $10^{-7}$M. The immediate and sustained (after 10 mins) effects of isoproterenol are shown. Bars represent mean ± S.E.M. of 5 experiments.
% CHANGE IN ATRIAL CONTRACTILE FORCE

Fig. 9
Figure 10. Reversibility of the negative inotropic effect of pr-MDI by ouabain (OUAB). Pr-MDI was added cumulatively resulting in a maximum negative inotropic effect at $10^{-4} \text{M}$. Thereupon, ouabain was added cumulatively to a final concentration of $10^{-6} \text{M}$. The immediate and sustained (after 10 mins) effects of ouabain are shown. Bars represent mean ± S.E.M. of 5 experiments.
% CHANGE IN ATRIAL CONTRACTILE FORCE

Fig. 10
negative inotropic effects of pr-MDI ($10^{-6} \text{M}-10^{-4} \text{M}$) upon the atrial preparations, and, respectively, the reversibility of these negative inotropic effects upon the addition of an excess calcium (2.5 mM above normal; Fig. 8), the addition of isoproterenol ($10^{-9} \text{M}-10^{-7} \text{M}$; Fig. 9), or the addition of ouabain ($10^{-7} \text{M}$ and $10^{-6} \text{M}$; Fig. 10). In each case, complete reversal of the negative inotropic effect of pr-MDI was achieved immediately upon introduction of each of the three myocardial stimulants, and atrial contractile force stabilized at the elevated stimulated level within 10 minutes.

**Effects of pr-MDI upon concentration-response relationships mediated by positive inotropic agents: Evaluation of membrane channel blocking activity**

Figure 11 depicts the effects of pr-MDI ($10^{-6} \text{M}$ to $3 \times 10^{-4} \text{M}$) on Ca$^{2+}$-mediated increases in contractile force in the atrial preparations. In 21 electrically-stimulated guinea pig left atrial preparations, basal tension developed in low calcium (0.5 mM) bathing solution at the end of the equilibration period was $159.8 \pm 25.9$ mg, with a threshold voltage of $1.87 \pm 0.12$ volts. Peak tension developed in response to Ca$^{2+}$ ion was generally achieved at 4-5 mM Ca$^{2+}$ with a dramatic decline in atrial force developed at concentrations exceeding 7 mM Ca$^{2+}$, probably due to the membrane stabilizing action of the excess calcium. Statistical analysis of the contractile responses of stimulated atria to calcium in the presence and absence of
Figure 11. Effect of pr-MDI on the concentration-response curve to calcium in isolated left atria. Each point on the control curve represents the mean ± S.E.M. of 21 experiments, while points on the remaining curves represent the mean ± S.E.M. of 4 experiments. Values are corrected for baseline shifts caused by the intrinsic negative inotropic action of pr-MDI. (*p < 0.05 as compared to control).
ATRIAL CONTRACTILE RESPONSE
(% OF MAXIMUM CONTROL RESPONSE)

CALCIUM CONCENTRATION (M)

Fig. 11
pr-MDI (after correcting for baseline shifts caused by the intrinsic negative inotropic effects of pr-MDI) indicates that concentrations of $10^{-6}$ M and $10^{-5}$ M pr-MDI resulted in no significant alteration in calcium-mediated positive inotropy at any calcium concentration along the curve (Fig. 4). The $10^{-4}$ M concentration of pr-MDI significantly depressed the contractile effects of calcium at only two points along the concentration-response curve, while the very high concentration of $3 \times 10^{-4}$ M pr-MDI resulted in significant depression of the contractile effects of calcium at several points along the curve with a shift of the curve to the right and a possible reduction of maximum (Fig. 11).

Figure 12 depicts the effects of pr-MDI ($10^{-6}$ M to $3 \times 10^{-4}$ M) on isoproterenol-mediated increases in force of contraction in the atrial preparations, after correcting for baseline shifts caused by the intrinsic negative inotropic effects of pr-MDI. In 22 electrically-stimulated guinea pig left atrial preparations, basal tension developed at the end of the equilibration period in normal physiological medium was $1123.0 \pm 89.7$ mg, with a threshold voltage of $2.00 \pm 0.16$ volts. Peak tension developed in response to isoproterenol was generally achieved at a concentration of $10^{-7}$ M. Statistical analysis of the contractile responses of the stimulated atria to isoproterenol in the presence and the absence of pr-MDI indicates that, with the exception of one point on the $10^{-6}$ M pr-MDI curve,
Figure 12. Effect of pr-MDI on the concentration-response curve to isoproterenol in isolated left atria. Each point on the control curve represents the mean ± S.E.M. of 22 experiments, while points on the remaining curves represent mean ± S.E.M. of 4 experiments. Values are corrected for baseline shifts caused by the intrinsic negative inotropic action of pr-MDI (*p < 0.05 as compared to control).
ISOPROTERENOL CONCENTRATION (M)

ATRIAL CONTRACTILE RESPONSE
(% OF MAXIMUM CONTROL RESPONSE)

FIG. 12

ISO

PROTERENOL CONCENTRATION (M)
concentrations of $10^{-6}$ and $10^{-5}$M pr-MDI did not have any effect on the positive inotropic effect of isoproterenol. Paradoxically, however, higher concentrations of pr-MDI ($10^{-4}$M and especially $3 \times 10^{-4}$M) significantly potentiated the contractile effect of isoproterenol.

Figure 13 depicts the effects of pr-MDI ($10^{-6}$M to $3 \times 10^{-4}$M) on ouabain-mediated increases in force of contraction in the atrial preparations after correcting for baseline shifts caused by the intrinsic negative inotropic effects of pr-MDI. In 23 electrically-stimulated guinea pig left atrial preparations, basal tension developed at the end of the equilibration period was $1422.8 \pm 111.6$ mg, with a threshold voltage of $1.78 \pm 0.16$ volts. Values on this figure are presented as a percent of the maximum control response to a challenge dose of $10^{-7}$M isoproterenol (see Methods). Peak tension developed in response to ouabain was generally achieved at a concentration of $3 \times 10^{-6}$M, with the preparations often failing to follow electrical stimulation upon prolonged exposure to $10^{-5}$M ouabain, and invariably failing to follow stimulation at higher concentrations. With the exception of the lowest points on the curves, statistical analysis revealed no significant differences in the concentration-response relationship to ouabain in the absence or the presence of pr-MDI ($10^{-6}$ to $3 \times 10^{-4}$M).
Figure 13. Effect of pr-MDI on the concentration-response curve to ouabain in isolated left atria. Each point represents the mean ± S.E.M. of 4-6 experiments. Values are corrected for baseline shifts caused by the intrinsic negative inotropic action of pr-MDI. (* p < 0.05 as compared to control).
ATRIAL CONTRACTILE RESPONSE
(% OF MAXIMUM CONTROL RESPONSE)

Fig. 13

ATRIAL CONTRACTILE RESPONSE
(% OF MAXIMUM CONTROL RESPONSE)

O M 3 M Pr-MDI
10^-5 M Pr-MDI
10^-4 M Pr-MDI
3 x 10^-4 M Pr-MDI

OUABAIN CONCENTRATION (M)

10^{-8}
10^{-7}
10^{-6}
10^{-5}
Comparison of the characteristics of negative inotropic actions of pr-MDI and inhibitors of mitochondrial energy production

In the absence of any cardiodepressant (Fig. 14), excess extracellular calcium (5 mM) produced a 104% increase in atrial force of contraction above the basal intrinsic force (n=4), which declined gradually within 10 mins in the continued presence of the high calcium concentration, but remained relatively stable at an elevated level of ~ 50% above the basal intrinsic force for the 30-min observation period. By contrast, cumulative addition of $10^{-9}$, $10^{-8}$, to $10^{-7}$M isoproterenol in the absence of any cardiodepressant produced 12%, 74%, and 162% increases in atrial force of contraction, respectively, above basal intrinsic force (n=4) which were not sustained (Fig. 14). The characteristics of the negative inotropic actions of pr-MDI and several inhibitors of mitochondrial energy production (rotenone, a Site I inhibitor of mitochondrial electron transport; antimycin A, a Site II inhibitor of electron transport; 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation) were investigated by comparing the characteristics of their reversal by addition of excess Ca$^{2+}$ or by the cumulative addition of isoproterenol. The profiles of the negative inotropic effects and the characteristics of reversal for each individual cardiodepressant are described below.
Figure 14. Characteristics of the positive inotropic effects of excess calcium (2.5 mM above normal) and of isoproterenol on the guinea pig left atrium. Mean ± S.E.M.
Fig. 14

% CHANGE IN ATRIAL CONTRACTILE FORCE

2.5 [10 +20 +30] MINS
CALCIUM (mM)

10^-6 10^-8 10^-7 [10 +20 +30] MINS
ISOPROTERENOL (M)
Figure 15 shows the concentration-dependent negative inotropic effect of $10^{-6}$-$10^{-4}$ M pr-MDI (n=13), and the characteristics of its reversal by high (5 mM) calcium levels (n=5) and by isoproterenol (n=8). High calcium levels reversed the negative inotropic effect of $10^{-4}$ M pr-MDI, resulting in a total increase in atrial force of contraction of 119% over the depressed level (-46%) induced by $10^{-4}$ M pr-MDI. This calcium-induced positive inotropy was sustained over the 30-min observation period, albeit at a reduced level during the last 20 mins of observation.

Cumulative addition of isoproterenol resulted in a concentration-dependent reversal of the negative inotropic effect of $10^{-4}$ M pr-MDI by concentrations of $10^{-9}$-$10^{-7}$ M of the cardiostimulant. The positive inotropic effect of the final concentration of $10^{-7}$ M isoproterenol was sustained for at least 10 mins (being 82% above the depressed level induced by $10^{-4}$ M pr-MDI; p < 0.05), but then declined altogether by 30 mins. This sustained response to isoproterenol was not observed with rotenone, antimycin A, or di-nitrophenol (see below).

Figure 16 shows the concentration-dependent negative inotropic effect of $10^{-8}$-$10^{-6}$ M rotenone (n=9), and the characteristics of its reversal by high (5 mM) calcium levels (n=4) and by isoproterenol (n=5). Elevated calcium caused a reversal of the negative inotropic effect of
Figure 15. Characteristics of the negative inotropic effect of pr-MDI on the guinea pig left atrium, and its reversal by excess calcium (2.5 mM above normal) and by isoproterenol. Mean ± S.E.M.
Fig. 15

% CHANGE IN ATRIAL CONTRACTILE FORCE

-100 -80 -60 -40 -20 0 20 40 60 80 100 120 140

10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}

PROPYL MDI (M)

2.5 [ +10 +20 +30 ] MINS

CALCIUM (mM)

10^{-9} 10^{-8} 10^{-7} [ +10 +20 +30 ] MINS

ISOPROTERENOL (M)
Figure 16. Characteristics of the negative inotropic effect of rotenone on the guinea pig left atrium, and its reversal by excess calcium (2.5 mM above normal) and by isoproterenol. Mean ± S.E.M.
Figure 16: Graph showing the percentage change in atrial contractile force with the change in concentrations of Rotenone (M), Calcium (mM), and Isoproterenol (M).
10^{-6}\text{M} \text{ rotenone. However, the total magnitude of this response to elevated calcium (which amounted to a 66\% increase in atrial contractile force above the depressed levels induced by 10^{-6}\text{M} \text{ rotenone; Fig. 16) was significantly smaller (p < 0.05) than the total magnitude of the response to this cation in presence of 10^{-4}\text{M} \text{ pr-MDI (which amounted to a 119\% increase over the depressed levels induced by 10^{-4}\text{M} \text{ pr-MDI; Fig. 15). Furthermore, the positive inotropic effect of elevated calcium in presence of rotenone was not sustained, with the force of atrial contractions returning back towards the depressed level produced by rotenone within 10 mins of exposure to elevated calcium. At all time points, the response to calcium in presence of rotenone (Fig. 16) was significantly less (p < 0.05) than the response to this cation in presence of pr-MDI (Fig. 15).}

The negative inotropic effect of 10^{-6}\text{M} \text{ rotenone was reversed in a concentration-dependent manner by 10^{-9}-10^{-7}\text{M isoproterenol. The total positive inotropic action produced by the final concentration of 10^{-7}\text{M isoproterenol in presence of 10^{-6}\text{M rotenone was 101\% above the depressed levels induced by 10^{-6}\text{M rotenone (Fig. 16), and was equivalent to that produced by a tenfold lower concentration of 10^{-8}\text{M isoproterenol in presence of 10^{-4}\text{M pr-MDI (a change of 90\%; Fig. 15). The positive inotropic effect of isoproterenol in presence of rotenone was transient, returning}
towards the depressed level produced by rotenone within 10 mins.

Figure 17 shows the concentration-dependent negative inotropic effect of $10^{-9} - 3 \times 10^{-6}$M antimycin A (n=10), and the characteristics of its reversal by high (5 mM) calcium levels (n=4) and by isoproterenol (n=6). Elevated calcium reversed the effect of $3 \times 10^{-6}$M antimycin A, but the total magnitude of the positive inotropic action of calcium (a 58% change from the depressed level produced by antimycin A; Fig. 17) was significantly smaller ($p < 0.05$) than the total magnitude of the response to calcium in presence of $10^{-4}$M pr-MDI (a 119% change from the depressed level induced by pr-MDI; Fig. 15). However, the reversal of antimycin A action by excess calcium resembled the reversal of pr-MDI action by this cation in being sustained ($p < 0.05$ at 10, 20, and 30 mins compared to the depressed levels induced by $3 \times 10^{-6}$M antimycin A; Fig. 17), albeit at significantly lesser magnitudes ($p < 0.05$) at all time points (compare with Fig. 15).

The negative inotropic effect of $3 \times 10^{-6}$M antimycin A was reversed in a concentration-dependent manner by $10^{-9} - 10^{-7}$M isoproterenol (Fig. 17), with the characteristics of this reversal closely resembling those of the reversal of the negative inotropic effect of $10^{-4}$M pr-MDI by isoproterenol (Fig. 15) except that they were more transient.
Figure 17. Characteristics of the negative inotropic effect of antimycin A on the guinea pig left atrium, and its reversal by excess calcium (2.5 mM above normal) and by isoproterenol. Mean ± S.E.M.
% CHANGE IN ATRIAL CONTRACTILE FORCE

Fig. 17

<table>
<thead>
<tr>
<th>Drug / Concentration</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimycin A (M)</td>
<td>10^-5</td>
</tr>
<tr>
<td>Calcium (mM)</td>
<td>2.5</td>
</tr>
<tr>
<td>Isoproterenol (M)</td>
<td>10^-6</td>
</tr>
<tr>
<td>Time (MINS)</td>
<td>140</td>
</tr>
</tbody>
</table>
Figure 18 shows the concentration-dependent negative inotropic effect of $10^{-7} - 3 \times 10^{-5}$M dinitrophenol (n=11), and the characteristics of its attenuation by high (5mM) calcium concentrations (n=7) and by isoproterenol (n=4). High calcium significantly reversed the negative inotropic effect of $3 \times 10^{-5}$M dinitrophenol ($p < 0.05$), but the magnitude of the peak positive inotropic effect of calcium (a 49% change from the dinitrophenol-depressed level) was significantly smaller ($p < 0.05$) than that produced by this cation in the presence of pr-MDI (a change of 119%; Fig. 15). Furthermore, the calcium-mediated reversal of this dinitrophenol-induced cardiodepression was very transient (<10 mins), with the magnitude of reversal being significantly less ($p < 0.05$) at all time points than the calcium-induced responses in the presence of pr-MDI (Fig. 15).

The negative inotropic effect of $3 \times 10^{-5}$M dinitrophenol was not affected by $10^{-9}$ or $10^{-8}$M isoproterenol, but was significantly ($p < 0.05$) and very transiently attenuated by $10^{-7}$M isoproterenol (Fig. 18). The magnitude of the peak positive inotropic effect of $10^{-7}$M isoproterenol (a 42% change above the dinitrophenol-depressed level) was roughly equivalent to that effect produced by $10^{-9}$M isoproterenol in the presence of pr-MDI (Fig. 15).
Figure 18. Characteristics of the negative inotropic effect of 2:4-dinitrophenol on the guinea pig left atrium, and its reversal by excess calcium (2.5 mM above normal) and by isoproterenol. Mean ± S.E.M.
Fig. 18
Intracellular Localization of Pr-MDI in the Isolated, Perfused Guinea Pig Heart

Cardiac effects in the isolated, perfused guinea pig heart

The effect of $3 \times 10^{-5}$M pr-MDI on cardiac force and rate of contraction of the isolated perfused guinea pig hearts is plotted as a function of time in Figure 19. Pr-MDI produced a negative inotropic effect that was immediate in onset, reaching a peak of 45% below control values at 35 mins of perfusion, with the magnitude of this inotropic difference between treated and untreated hearts being maintained for the remaining 10 mins of perfusion time. Cardiac chronotropy, on the other hand, was not affected by pr-MDI.

Tissue uptake and washout

The effluent concentrations of pr-MDI, calculated from the $^{14}$C content of effluent aliquots for various time periods during the 45-min cardiac perfusion with $^{14}$C-pr-MDI and subsequent 45-min washout of radioactivity with drug-free medium, are plotted as a function of time in Figure 20. During the 45 mins of perfusion with $3 \times 10^{-5}$M pr-MDI (30 nmole/ml), a steady state is essentially achieved by 35-45 mins, at which time the hearts are extracting approximately 45% of the perfusing drug with 55% of the drug appearing in the effluent. On the other hand, an equilibrium condition (with equality
Figure 19. Time course of the effects of $3 \times 10^{-5} \text{M pr-MDI}$ on inotropy and chronotropy of the isolated perfused guinea pig heart. Each point represents the mean ± S.E.M. of 8 preparations.
Fig. 19
Figure 20. Time course for the uptake and washout of $3 \times 10^{-5}$M pr-MDI (30 n mole/ml, with $^{14}$C-pr-MDI) from isolated perfused guinea pig hearts. Each point represents the mean ± S.E.M. of 4 preparations.
Fig. 20
between the infusate concentration of 30 n mole/ml of pr-MDI and the steady state effluent concentration of the drug) is not established by the end of the 45-min drug perfusion period. During the 45-min washout period, MDI concentrations in the effluent were fitted to both a monoexponential and a biexponential model with the aid of the NONLIN computer program (Metzler et al., 1974). Visual inspection of the distribution of experimental points about the theoretical lines (Figure 21) and comparison of the sum of squared deviations (Table 9) suggest that these drug concentrations decline in a biphasic rather than a monophasic manner. The first washout phase is a rapid, non-linear exponential phase occurring at the onset of the washout and lasting approximately 5 mins. This is followed by a second, log-linear washout phase lasting throughout the remainder of the 45-min washout period. In general, the washout portion of figure 20 tends to indicate a rather extensive loss of MDI from the heart over the 45 mins of washout. The results obtained in this section of the study were used to establish the parameters of drug perfusion and washout for the subsequent examination of tissue uptake and subcellular distribution of pr-MDI.
Figure 21. Fitting of pr-MDI washout data from Figure 20 to monoexponential (left) and biexponential (right) models by means of NONLIN computer program (Metzler et al., 1974).
Fig. 21
Table 9. Sums of squared deviations of pr-MDI washout data points from theoretical, computer-generated monophasic and biphasic lines.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Type of Theoretical Line</th>
<th>Sums of Squared Deviations of Data Points from Theoretical Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monophasic</td>
<td>8.000</td>
</tr>
<tr>
<td>Biphasic</td>
<td>0.041</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Theoretical lines were generated with the aid of the NONLIN computer program (Metzler \textit{et al.}, 1974)
Characterization of subcellular fractions

Ventricular subcellular fractions obtained by the differential and density gradient centrifugation protocol described under Methods were examined for relative purity by assays of marker enzymes. Six non-perfused guinea pig hearts were individually homogenized and the subcellular fractions characterized enzymatically. Additionally, five guinea pig hearts were perfused with $3 \times 10^{-5}$M non-radio-labelled pr-MDI, and then immediately homogenized (without a washout period), and the subcellular fractions characterized enzymatically. The purpose of omitting the washout of pr-MDI prior to homogenization was to ensure the maximum presence of the drug during the subsequent subcellular fractionation steps, and thus maximize the detection of any drug-induced changes in enzyme distribution or activity that would confound the interpretation of subsequent results involving the subcellular distribution of radio-labelled drug. The mean enzyme activities and protein contents of the subcellular fractions and the overall enzyme activity profiles for the non-perfused and MDI-perfused hearts were found to be remarkably similar, indicating that the 45-min perfusion with $3 \times 10^{-5}$M pr-MDI had minimal effects upon the enzyme activities monitored or the fractionation protocol itself. Figure 22 depicts the pooled enzyme activity profiles for the subcellular fractions from the 11 guinea pig hearts, for each of the
Figure 22. Distribution of marker enzyme activities in subcellular fractions of guinea pig ventricles. Each bar represents the mean ± S.E.M. of 11 preparations. Numbers within the bars represent purification indices (ratio of enzyme activity in a given fraction to that of the homogenate); the indices were omitted if they were 0.13 or less. H = homogenate; N = nuclei/cell debris; M = mitochondria; S = supernatant/cytosol; I₁, I₂ and I₃ = sucrose gradient interfaces 1, 2 and 3; Pi = inorganic phosphate.
Fig. 22
following marker enzymes: succinate cytochrome c-reductase, a constituent of mitochondrial particles (Green and Burkhardt, 1962); NADPH cytochrome-c-reductase, a putative marker for sarcoplasmic reticulum (Kidwai et al., 1971; Tibbits et al., 1981); total (Mg^{++} + Na^{+} + K^{+})ATPase, and Na^{+}/K^{+} ATPase, a commonly used marker enzyme for sarcolemma (Kidwai et al., 1971; Hui et al., 1976; Collins and Cook, 1981).

As evident from the enzyme distribution pattern in Figure 22, the mitochondrial fraction shows the highest specific activity of succinate cytochrome-c-reductase. The specific activity of the putative sarcoplasmic reticulum marker, NADPH cytochrome c-reductase, is highest in the three interface fractions harvested from the discontinuous sucrose density gradient (see Methods), with a suggestive higher relative enrichment in interface 1. The total ATPase activity, as expected, was moderate in the mitochondrial fraction and high in the three interface fractions harvested from the sucrose gradient. The sarcolemmal marker enzyme, Na^{+}/K^{+} ATPase, shows a significantly higher specific activity (p< 0.01) in interface 2 than in interfaces 1 or 3 harvested from the sucrose gradient.

**Tissue concentration of pr-MDI**

Figure 23 shows the pr-MDI content of atrial and ventricular tissues after perfusion of isolated guinea pig hearts for various periods of time with 3 x 10^{-5} M
Figure 23. Time course of pr-MDI accumulation by atrial and ventricular tissue from guinea pig isolated hearts perfused with $3 \times 10^{-5} M$ pr-MDI with $^{14}$C-labeled drug. Each point represents the mean ± S.E.M. of 3-4 preparations. Numbers in parentheses represent simple tissue to medium ratios. The corresponding tissue to medium ratios for the different time points calculated according to the assumptions of Lorenzo and Spector (1973) are:

<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>Atrium</th>
<th>Ventricle</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>61.00</td>
<td>79.74</td>
</tr>
<tr>
<td>15</td>
<td>50.98</td>
<td>65.51</td>
</tr>
<tr>
<td>30</td>
<td>65.02</td>
<td>82.74</td>
</tr>
<tr>
<td>45</td>
<td>59.32</td>
<td>76.04</td>
</tr>
</tbody>
</table>
pr-MDI containing $^{14}$C-pr-MDI tracer. It is evident that marked quantitative increases in the tissue retention of pr-MDI in both the atria and ventricles occur throughout the drug perfusion period. Ventricular tissue consistently displayed a greater retention of drug than atrial tissue at all time periods tested. Simple tissue/medium ratios (shown on Figure 23 in parentheses for each time point), as well as tissue/medium ratios calculated according to the assumptions of Lorenzo and Spector (1973) (shown in the legend to Figure 23), are quite high. This is consistent with a steady state uptake well below equilibrium conditions. The tissue/medium values for ventricular tissue are slightly greater than the corresponding values for atrial tissue, reflecting the greater accumulation of pr-MDI in ventricular tissue.

**Subcellular distribution of pr-MDI**

Figure 24 depicts the concentration of pr-MDI in the ventricular subcellular fractions obtained from guinea pig hearts perfused for various time periods with $3 \times 10^{-5}$M pr-MDI (containing $^{14}$C-pr-MDI tracer). The high content of pr-MDI (calculated as nMoles drug/mg protein) in the $109,000 \times g$ supernatant (cytosolic) fraction. The pr-MDI content of the $1085 \times g$ nuclear/cell debris fraction is a reflection of the presence of sheared cells in this fraction, as evidenced by activities of succinate
Figure 24. Distribution of pr-MDI in subcellular fractions of guinea pig ventricular tissue obtained from isolated hearts perfused with $3 \times 10^{-5}$M pr-MDI (with $^{14}$C-labeled drug). Each point represents the mean ± S.E.M. of 3-4 preparations.
Supernatant vs Homogenate vs Nuclei/Cell Debris

Fig. 24

Propyl MDI Concentration (nMole/mg Protein)

TIME OF PERFUSION (min)

MITOCHONDRIA
INTERFACE 1
INTERFACE 2
INTERFACE 3

Fig. 24
cytochrome c-reductase, NADPH cytochrome c-reductase, and total ATPase that are essentially equal to those of the total homogenate (see Figure 22). Likewise, the time-dependent increase in pr-MDI content in the nuclear/cell debris fraction and in the total homogenate fraction (Figure 24) parallels the time-dependent increase in drug retention in whole ventricular tissue (see Figure 22). Of greater interest in Figure 24 are the pr-MDI concentration profiles for the 14,500 x g mitochondrial fraction and the three 112,000 x g interface fractions harvested from the sucrose density gradient. While all four of these fractions display a time-dependent increase in pr-MDI concentration, the mitochondrial fraction and the interface 1 fraction clearly accumulate more drug ($p < 0.05$ or better at the 15 and 20 min time periods) than the interface 2 and 3 fractions. The mean recovery of radioactivity calculated in all experiments was $95.3 \pm 1.4\%$, and the mean recovery of the protein in the same experiments was $87.2 \pm 0.9\%$. These recovery values correspond to those reported by Marzo et al. (1977).
CHAPTER IV

DISCUSSION

Evaluation of MDIs in In Vivo Models of Arrhythmia

Antiarrhythmic activities of the tertiary pr- and bu-MDIs, as well as that of the quaternary ammonium analogue Q-bu-MDI, were evaluated in several models of experimental arrhythmia, with different electrophysiologic and ionic bases. Specifically, the MDIs were evaluated in models in which arrhythmias were induced by excess Ca$^{2+}$ in dogs and rats, aconitine in rats, methacholine in rats and chloroform/hypoxia in mice.

Both the tertiary and quaternary MDIs afforded significant protection against arrhythmias induced by excess calcium ion and by chloroform/hypoxia. The mechanism for the production of arrhythmias by excess calcium is considered to be due to the combined effects of decreased conduction velocity, decreased or complete loss of excitability in certain areas of the myocardium, and shortened refractory period in the ventricles (Brooks et al., 1955; Chung, 1977). Calcium ion is also thought to play a central role in the causation of chloroform/hypoxia-induced arrhythmias. It is thought, in the
chloroform/hypoxia assay, that the myocardium becomes sensitized to the dysrhythmogenic actions of endogenous catecholamines (Lawson, 1968), resulting in the facilitation of slow calcium-dependent action potential propagation in ischemic myocardium (Bowman and Rand, 1980). Such slow calcium-dependent action potentials have low propagation velocities and may contribute to the appearance of unidirectional block and reentry phenomena, which are of importance in the genesis of arrhythmias (Andersson, 1978).

In contrast to experimental arrhythmias induced by excess calcium or chloroform/hypoxia, calcium ions do not appear to play a central role in the causation of aconitine- or methacholine-induced arrhythmias. Voltage clamp experiments suggest that delayed repolarization resulting from impaired closure of sodium channels and consequent prolongation of sodium conductance underlies the arrhythmogenic action of aconitine (see Bowman and Rand, 1980). The mechanism of arrhythmogenic action of the cholinergic agonist methacholine is, at the cellular level, not completely understood, though it is thought that an increase in the cardiac plasma membrane permeability to potassium plays the central role in the alterations in the electrical properties of the cardiac cell (plasma membrane hyperpolarization leading to fall in
prepotential, fall in pacemaker rate, rapid repolarization, reduced action potential duration and refractory period) which lead to a decrease in conduction rate in response to a cholinergic agonist (see Bowman and Rand, 1980). The protective activities of both the tertiary and quaternary MDIs against arrhythmias induced by aconitine and methacholine were suggestive but not impressive; that is, while, in both evaluations, the MDIs displayed protective trends, they only displayed barely significant protection at one dose each for the Q-bu-MDI and pr-MDI against aconitine-induced arrhythmias, and failed, at all doses for all three analogues, to display statistically significant protection against methacholine-induced arrhythmia. Interestingly, in the latter case, the MDIs did not appear to exacerbate methacholine-induced atrio-ventricular (A-V) dissociation, which a calcium entry blocker would be expected to do (See below).

Hence, in several models of experimental arrhythmia in several animal species, both the tertiary and quaternary MDIs display at least suggestive protective activity and, in the cases of arrhythmias induced by calcium ion, chloroform/hypoxia and, for the tertiary MDIs, ouabain (Piascik et al., 1979b), significant protective activity. Interestingly, in those evaluations in which the tertiary MDIs and the quaternary analogue were both examined, the
Q-bu-MDI was always found to exhibit far greater protective activity (on a mg/kg basis) than its uncharged tertiary analogues or, for that matter, the standard agents included in the evaluations. This finding is of timely interest, in that a great amount of attention has recently been called to the important antiarrhythmic/antifibrillatory effects of quaternary ammonium compounds such as bretylium and pranolium (Lucchesi, et al., 1982).

The ability of the MDI analogues to provide significant protection against calcium-dependent arrhythmias, coupled with their unimpressive effects against aconitine- and methacholine-induced arrhythmias, supports the hypothesis that the methylenedioxyindene compounds may interfere in some manner with Ca\textsuperscript{2+} availability or action. The precise mechanism by which the MDIs produce antiarrhythmic effects cannot be conclusively drawn from these studies and, for that matter, the precise mechanism by which "calcium antagonists," for the most part calcium entry blockers, produce antiarrhythmic effects has not been conclusively established. The antiarrhythmic activities of calcium entry blockers against experimental and clinical arrhythmias are well documented (Bergey et al., 1981; Ellrodt et al., 1980), with the most significant antiarrhythmic effect of the drug class generally accepted as being its direct electrophysiological actions,
(interference with slow inward calcium current), particularly delay in A-V nodal conduction (due to inhibition of slow inward calcium current in the atrioventricular conducting fibers, whose action potentials are nearly totally dependent on calcium current), which is thought to be the fundamental mechanism by which the ventricular response in atrial fibrillation and flutter is controlled and A-V nodal reentrant paroxysmal supraventricular tachycardia is abolished (Heng et al., 1975; Ellrodt et al., 1980). The added ability of calcium entry blockers to inhibit fast inward sodium current is considered by some to be of significant importance in the antiarrhythmic effects of these compounds (Henry, 1979). More recently, however, attention has been called to the possibility that "calcium antagonists" might favorably influence arrhythmias indirectly by influencing myocardial homeostasis (particularly Ca\(^{2+}\) homeostasis) in such a way as to preserve myofiber structure and function (Ellrodt et al., 1980). It is generally accepted that alterations in Ca\(^{2+}\) homeostasis play a critical role in myocardial ischemic injury (Trump et al., 1976; Katz and Reuter, 1979), and it has also been well demonstrated that in zones of ischemically injured myocardium, the natural stimulation of cells leads to the propagation of slow calcium-dependent action potentials, which possess low propagation velocity and hence
may lead to arrhythmia, as previously discussed in the case of chloroform/hypoxia-induced arrhythmias (Andersson, 1978). Given that "calcium antagonists" possess pharmaco-logical properties which might tend to protect the ischemic myocardium and preserve cell structure and function (Grinwald and Nayler, 1981; Schwartz et al., 1981), it is reasonable to attribute part of the antiarrhythmic efficacy of "calcium antagonists" to a favorable effect upon intracellular calcium homeostasis. In fact, a recent study on the antiarrhythmic activity of verapamil, an antiarrhythmic calcium entry blocker, describes the ability of the compound to protect against arrhythmias caused by an increase in cytosolic Ca$^{2+}$ concentration secondary to a reduction in mitochondrial Ca$^{2+}$ binding (Sugiyama et al., 1981). In this latter case, antiarrhythmic efficacy is due to a favorable effect upon cytosolic Ca$^{2+}$ levels by verapamil rather than the electrophysiological event which precedes the lowering of cytosolic Ca$^{2+}$ concentration.

With regard to the mechanism of antiarrhythmic action of the MDIs, it is reasonable to expect that the compounds, particularly the tertiary pr- and bu-MDIs which have been characterized as intracellular calcium antagonists, might exert an antiarrhythmic effect through a favorable influence upon intracellular Ca$^{2+}$ homeostasis. Supporting this point, the tertiary bu-MDI has been shown
to inhibit the swelling of cardiac mitochondria induced by inorganic phosphate (Matlib et al., 1981). This is a very relevant finding since recent evidence suggests that ischemia of myocardial tissue (which, as has been discussed above, plays an important role in the causation of dysrhythmia) results in an increase in inorganic phosphate concentration which induces mitochondrial swelling and uncoupling of oxidative phosphorylation by stimulating energy-dissipating intramitochondrial cycling of Ca^{2+} (Schwartz et al., 1981). An inhibition of such swelling would preserve mitochondrial structure and function, contributing in turn to a preservation of cell function during ischemia. It may certainly not be precluded, however, that the electrophysiological effects of the compounds, which are reported to nonselectively inhibit both fast sodium-dependent and slow calcium-dependent inward currents (Rahwan et al., 1981; Muir, 1982) contribute to antiarrhythmic efficacy.

**Evaluation of the MDIs in Isolated Guinea Pig Atrial Preparations**

The evaluation of the tertiary pr- and bu-MDIs (which have been characterized as intracellular calcium antagonists, based upon earlier evaluations of the compounds in various smooth and skeletal muscle systems as well as in the bovine adrenal medulla model for stimulus-secretion coupling) as well as the quaternary ammonium
Q-bu-MDI (which, by virtue of its charge, is expected to be excluded from intracellular space) in isolated cardiac muscle preparation allows for a fairly comprehensive examination of the pharmacological effects of the compounds on cardiac muscle, but also allows for an investigation of the role played by calcium entry blockade in the pharmacological actions of the tertiary MDI.

A quantitative difference clearly exists between the effects of the tertiary MDIs (pr-MDI and bu-MDI) and the quaternary analogue (Q-bu-MDI) on the mechanical and electrical activity of isolated guinea pig atria. The tertiary analogues display moderate negative inotropic effects, significantly depress the frequency-force curve of stimulated left atria at high concentrations, and significantly decrease membrane excitability at high concentrations (as reflected by the increase in threshold voltage required to drive the atria). The quaternary analogue, on the other hand, exhibits only mild negative inotropic effects at low concentrations, while positive inotropy is observed at the highest concentrations. The Q-bu-MDI does not influence the ability of the atria to follow high frequency stimulation, does not depress the frequency-force curve of stimulated left atria, and does not increase the threshold voltage required to drive the left atria. Both the quaternary and tertiary analogues
display little effect on atrial rate at lower concentrations, while at higher concentrations the tertiary analogues are more potent than the quaternary analogue in reducing atrial rate.

From a mechanistic point of view, the observation from the evaluation described above which is of the greatest interest is that while the tertiary and quaternary MDIs all produce mild negative chronotropic effects at high concentrations (very likely a reflection of calcium entry blockade at these high concentrations), the tertiary pr- and bu-MDIs display moderate negative inotropic effects in a lower concentration range, while the Q-bu-MDI displays relatively little effect upon inotropy. It is well documented that the calcium entry blocker-type "calcium antagonist" produces negative chronotropic effects in the same dosage range that depresses inotropy (Fleckenstein, 1977), since both the negative inotropic and negative chronotropic actions of the calcium entry blockers are due to the same action, inhibition of slow calcium-dependent inward current. The effects of the tertiary and quaternary MDIs upon inotropy and chronotropy, therefore suggest an additional, intracellular mechanism of negative inotropic action for the tertiary pr- and bu-MDIs, which the Q-bu-MDI does not share in this concentration range, perhaps because it is excluded from the intracellular space due
to its charge. Such an additional, intracellular site of negative inotropic action for the tertiary MDIs is also suggested by the finding that the tertiary MDIs can uncouple contraction in canine papillary muscle in the absence of effects upon action potential characteristics (Muir, 1982).

Other observations from the above evaluation (pharmacological effects at higher concentrations of the tertiary and quaternary MDIs) are quite consistent with and provide physiological correlates for the concurrently reported electrophysiological effects of the MDIs in cardiac muscle (Rahwan et al., 1981; Muir, 1982). It is likely that the mild, negative chronotropic activities of the tertiary and quaternary MDIs (observed at concentrations of $3 \times 10^{-5}$ M or greater in guinea pig atria) are a reflection of the ability of the compounds, at these higher concentrations, to inhibit slow calcium-dependent inward current, since action potentials in pacemaker cells are nearly totally dependent upon calcium current (Sobel, 1981). Similarly, the ability of the tertiary MDIs (at $3 \times 10^{-4}$ M) to reduce following capacity is consistent with their prolongation of action potential duration in working myofibers (Bowman and Rand, 1980); and the ability of the tertiary MDIs (at $3 \times 10^{-4}$ M) to reduce membrane excitability may be a reflection of their ability to inhibit fast
sodium-dependent inward current at the concentration (Klein et al., 1960; Landmark, 1972a). The depression of the frequency-force profile by the tertiary MDIs (at $3 \times 10^{-4}$ M) is not as easily interpreted. While frequency-force relationship of isolated cardiac muscle is subject to depression both by calcium entry blockers (Chiba, 1976; Chiba et al., 1978) and by agents which interfere with fast, sodium-dependent current (Mensing and Hilgemann, 1981), it may not be precluded, due to the critical role played by intracellular Ca\(^{2+}\) in the initiation and regulation of contracile force, that an interference with Ca\(^{2+}\) action and/or availability contributes to this effect.

A final point of interest regarding the evaluation described above is the inability of the Q-bu-MDI (at $3 \times 10^{-4}$ M, equimolar to the tertiary analogues) which is reported to share most of the electrophysiological properties possessed by the tertiary MDIs, to produce effects similar to those of the tertiary analogues upon following capacity, membrane excitability and frequency-force profile. Such a dichotomy in effect between the charged Q-bu-MDI and the uncharged tertiary MDIs might indicate that membrane electrophysiological properties alone are not responsible for the effects upon mechanical and electrical activity or, alternatively, may simply reflect a quantitative difference in potency between the tertiary
and quaternary compounds for those electrophysiological properties responsible for the observed effects.

In order to more fully examine the mechanism of negative inotropic action of the tertiary MDIs, the characteristics of reversal of the negative inotropic action of pr-MDI was investigated. The negative inotropic effect of pr-MDI is readily reversed, or functionally antagonized, by the addition of excess Ca$^{2+}$, isoproterenol or ouabain. Excess extracellular Ca$^{2+}$ is thought to stimulate cardiac contraction by increasing slow calcium-dependent inward current (Carmeleit and Vereeke, 1969). Beta-adrrenergic agonists such as isoproterenol stimulate cardiac excitation-contraction coupling by indirectly augmenting entry of extracellular calcium through the slow inward calcium channels (Zipes et al., 1975; Langer, 1980; Wollenberger and Will, 1978) subsequent to activation of adenylate cyclase and eventual cyclic-AMP-mediated phosphorylation of sarcolemmal proteins (Wollenberger and Will, 1978). On the other hand, most of the published evidence argues against an influence of digitalis glycosides on the slow inward calcium current (Beeler and Reuter, 1970; New and Trautwein, 1972; Greenspan and Morad, 1975; McDonald et al., 1975) and favors a mechanism of increased calcium influx through other membrane channels resulting from an alteration of the movement of a sarcolemmal Na$^+$/Ca$^{2+}$ exchanger.
transport system secondary to an increase in intracellular sodium concentration (Langer, 1980). The reversibility of the negative inotropic effect of pr-MDI with these positive inotropic interventions satisfies a key criterion for classification of a drug as a "calcium antagonist," as set forth by Fleckenstein (Fleckenstein et al., 1975; Fleckenstein, 1977). It has been pointed out, however, that the satisfaction of this criterion alone does not provide sufficient evidence for classification of a drug as a "calcium antagonist," nor does it provide information about the precise mechanism by which a drug may be interfering with Ca$^{2+}$ action or availability (Nayler and Poole-Wilson, 1981).

In order to test the hypothesis of Weishaar et al., (1982), that the cardiodepressant action of the tertiary MDIs is due to an interference with energy production by the mitochondria rather than through an effect upon intracellular Ca$^{2+}$ movements per se, the characteristics of reversal of the negative inotropic action of pr-MDI by excess Ca$^{2+}$ and by isoproterenol were compared to the characteristics of reversal of comparable cardiodepressions produced by 2,4-dinitrophenol (an uncoupler of mitochondrial oxidative-phosphorylation), rotenone (a Site I inhibitor of electron transport), and antimycin A (a Site II inhibitor of electron transport). A discussion
of the characteristics of reversal for the aforementioned cardiodepressants follows.

The mechanisms by which excess Ca$^{2+}$ and isoproterenol stimulate cardiac contraction have been discussed above. In the absence of cardiodepressant agents, raising the extracellular calcium concentration to 5 mM resulted in a sustained increase in atrial force of contraction. On the other hand, the positive inotropic effect of isoproterenol (10$^{-9}$-10$^{-7}$M) in the absence of a cardiodepressant was not sustained. The transient effect of isoproterenol is not due to neuronal (uptake-1) or extraneuronal (uptake-2) mechanisms for terminating sympathomimetic drug action since this β-receptor agonist is not taken up by adrenergic neurones (Bowman and Rand, 1980) and, in contrast to other species and tissues, extraneuronal uptake is poorly developed in the guinea pig myocardium (Bönisch and Trendelenburg, 1974; Ebner, 1981). It is possible that the transient nature of the action of isoproterenol is due to chemical oxidation of this agent in the tissue bath since an antioxidant was not used in these experiments, although other possibilities (desensitization, fade phenomenon) cannot be excluded.

The demonstration of a negative inotropic effect of pr-MDI in this evaluation is consistent with previous similar observations in the rabbit Langendorff (Piascik
et al., 1979a) and in isolated guinea pig atria (discussed above), and so is the demonstration of the sustained reversibility of this effect by increasing extracellular calcium concentration or by adding isoproterenol (satisfaction of the Fleckenstein reversibility criterion, discussed above). It is not known at the present time why the cardiostimulant action of isoproterenol is more prolonged in the presence of $10^{-4}$M pr-MDI than in its absence, but, as we shall see below, this is a reproducible effect.

Although metabolic inhibitors such as dinitrophenol inhibit slow calcium channel transport (Sperelakis and Schneider, 1976), their effects can be accounted for in terms of inhibition of the energy requirements needed for maintaining the configurational state of the cell membrane compatible with the maintenance of normal slow channel ultrastructure and function (Sperelakis and Schneider, 1976; Nayler, 1982). Since dinitrophenol is an uncoupler of mitochondrial oxidative-phosphorylation, which allows electron transport (respiration) to continue without concomitant generation of ATP (see Olson, 1982), it would be expected to block calcium influx through the energy-requiring slow calcium channels (Nayler and Grinwald, 1981). Indeed, neither high extracellular calcium concentrations nor the highest concentration of isoproterenol ($10^{-7}$M) were capable of producing more than a transient
attenuation of the negative inotropic effects of dinitrophenol. These obtunded effects are consistent with a further depletion induced by calcium and isoproterenol of the already compromised energy stores of a myocardium poisoned by dinitrophenol. Hence, the characteristics of reversal of dinitrophenol-induced cardiodepression contrast sharply with those of pr-MDI-induced cardiodepression, inconsistent with the hypothesis that the MDIs may act as uncouplers of oxidative-phosphorylation (Weishaar et al., 1982).

The arrangement of the mitochondrial electron transport carriers (see Olson, 1982) is such that electrons of reducing equivalents extracted from substrates in the tricarboxylic acid cycle, the fatty acid β-oxidation sequence, and indirectly from glycolysis are fed into the electron transport chain at the level of NADH or coenzyme Q from the primary NAD⁺- and FAD-lined dehydrogenase reactions, and are transported to molecular oxygen through the cytochrome chain, with coupled generation of ATP at three sites. Rotenone inhibits the electron transfer chain at the level of the flavoprotein, NADH dehydrogenase (site I). Hence, electrons or reducing equivalents derived from NAD⁺-linked dehydrogenases are not oxidized by a rotenone-inhibited respiratory chain, whereas those derived from flavin-linked dehydrogenases are freely oxidized with the
coupled generation of ATP at the two uninhibited sites (Sites II: cytochrome c reductase, and Site III: cytochrome oxidase). On the other hand, the antibiotic antimycin A inhibits cytochrome c reductase and electron transfer at the level of cytochrome b (Site II) behind the crossover point where electrons or reducing equivalents feed into the electron transport chain. This leads to blockage of electron transfer and impairment of energy (ATP) generation by the electron transport chain.

As anticipated from the above discussion, rotenone was unable to completely block the positive inotropic actions of high calcium levels or isoproterenol. However, the total magnitude of the calcium-induced response in presence of rotenone was greatly diminished and transient as compared to the response to calcium in the presence of pr-MDI. The responses to isoproterenol in presence of rotenone were similarly attenuated. The blocking effects of rotenone are therefore intermediate between those of pr-MDI and dinitrophenol. This is consistent with the continued generation of ATP at Sites II and III of the electron transport chain in presence of rotenone, thus allowing the continued (albeit reduced) function of the slow calcium channels.

The results obtained with calcium and isoproterenol in presence of antimycin A were similar in many respects to those obtained in presence of rotenone. Thus,
the total magnitude of the calcium-induced positive inotropic effect on the atria in presence of antimycin A was significantly obtunded when compared with the response to this cation in presence of pr-MDI, whereas the isoproterenol-induced response in presence of antimycin A was not remarkably different from that observed in presence of pr-MDI. Such findings are not consistent with a complete shutdown of the electron transport chain by antimycin A. The failure of antimycin A to produce effects more closely resembling those produced by dinitrophenol than by rotenone may be ascribed to the relatively low final concentration of $3 \times 10^{-6}$ M antimycin A used in our experiments, which was dictated by the necessity to titrate the concentrations of all four cardiodepressants to obtain an approximately 50% inhibition of atrial force of contraction with each agent. Noack and Greeff (1971), on the other hand, used a concentration of $5 \times 10^{-4}$ M antimycin A in a preparation of isolated mitochondria from rabbit heart in order to completely block the electron transport chain. Additionally, isoproterenol is capable of stimulating glycogenolysis and the tricarboxylic acid cycle in cardiac muscle, thereby increasing the rate of substrate-level phosphorylation with generation of some ATP (which is not subject to inhibition by electron transfer blockers or uncouplers of oxidative-phosphorylation) and provision of reducing equivalents to the
electron transport chain which might overcome the inhibitory action of electron transfer blockers.

Taken together, the present findings regarding the characteristics of reversal of rotenone and antimycin A, despite their limitations, differ substantially from those obtained with pr-MDI, and therefore do not support the claim (Weishaar et al., 1982) that the MDIs inhibit cardiac mitochondrial electron transport.

Findings in isolated cardiac muscle discussed thus far suggest that the tertiary MDIs possess a site and mechanism of negative inotropic action apart from calcium entry blockade at the sarcolemma. This additional mechanism of negative inotropic action does not appear to involve interference with mitochondrial energy production. To further investigate the possible existence of an additional mechanism of negative inotropic action of the tertiary MDIs apart from calcium entry blockade, the effects of pr-MDI upon concentration-response (force) relationships in isolated left atrial preparations were evaluated. A discussion of these effects as well as the rationale for the analysis follows.

The findings of Bristow and Green (1977) indicate that agents which are characterized primarily as calcium entry blockers antagonize the action of calcium in a nearly competitive manner in analyses of calcium-mediated concentration-response relationships in isolated cardiac
tissues. Thus, verapamil and methoxyverapamil, which are classified as slow calcium channel blockers (Kohlhardt et al., 1972a; Fleckenstein, 1974), produce nearly parallel shifts to the right of the calcium concentration-response curve without reduction of the maximum, when the concentration of these agents is less than $10^{-5}$ M (Bristow and Green, 1977). At higher concentrations, however, both compounds produce a decrease in slope and maximum of the calcium concentration-response curve (Bristow and Green, 1977). In the present investigation, pr-MDI at concentrations which produce negative inotropic effects ($10^{-6}$-$10^{-4}$ M) essentially did not alter the calcium concentration-response curve. These findings argue against any slow inward calcium channel blocking action of pr-MDI. Only at the very high concentration of $3 \times 10^{-4}$ M did this MDI cause a shift of the calcium curve to the right, indicative of possible slow calcium channel blocking activity, or perhaps nonspecific effects, at this high concentration.

The above conclusion concerning the lack of blocking effect of pr-MDI on the slow calcium channels is supported by the practical lack of effect of $10^{-6}$ and $10^{-5}$ M pr-MDI on the concentration-response curve to isoproterenol, despite the evident negative inotropic effects of the MDI at these concentrations. Higher concentrations of pr-MDI ($10^{-4}$ and $3 \times 10^{-4}$ M) caused a paradoxical potentiation of the positive inotropic effect of isoproterenol.
Bristow and Green (1977) demonstrated that the slow calcium channel blockers (verapamil and methoxyverapamil) clearly display noncompetitive antagonism in an analysis of concentration-response relationships mediated by isoproterenol.

Furthermore, pr-MDI ($10^{-6}$ to $3 \times 10^{-4}$ M) did not alter the ouabain concentration-response curve, indicating lack of inhibitory effects of this MDI on any presumptive membrane calcium entry routes mediating the positive inotropic effect of the cardiac glycoside.

This latter evaluation rules out sarcolemmal calcium entry blocking activity at lower, negative inotropic concentrations of pr-MDI (up to $3 \times 10^{-4}$ M), thereby suggesting the existence of an additional, presumably intracellular mechanism of negative inotropic action, confirming the interpretations of the previously discussed evaluations. Furthermore, the indication of calcium entry blockade at the high ($3 \times 10^{-4}$ M) concentration of pr-MDI is most consistent with the tertiary MDI display of mild negative chronotropic effect and the electrophysiological manifestation of interference with slow calcium-dependent inward current at concentrations above the range for negative inotropic effects.
Intracellular Localization of Pr-MDI in the Isolated, Perfused Guinea Pig Heart

Studies were undertaken in the isolated, perfused guinea pig heart to determine if the characteristics of tissue uptake and washout, tissue concentration and subcellular distribution of pr-MDI (with $^{14}$C-pr-MDI as radiotracer) is consistent with association or availability of the drug to an intracellular site (as suggested by the findings in isolated atrial preparations, discussed in the preceding section, and suggested by studies in other pharmacological test systems, reviewed in the Introduction).

Perfusion of isolated guinea pig hearts with $3 \times 10^{-5}$M pr-MDI produces a marked negative inotropic effect, with relatively no effect upon chronotropy; findings consistent with observations in the rabbit Langendorff (Piascik et al., 1979a) and isolated guinea pig atria (discussed in previous section), and, based upon the discussion in the previous section, may reflect negative inotropic action by a mechanism other than calcium entry blockade (and therefore perhaps at a non-membrane, intracellular site). The time course of the negative inotropic action of pr-MDI corresponds to the time course of myocardial tissue uptake and accumulation of this drug. Thus, the maximum magnitude of the negative inotropic action of pr-MDI occurred at about 35 minutes of drug perfusion.
which corresponds to the time of steady state uptake of 
the drug in whole hearts and to the high levels and 
tissue/medium ratios of pr-MDI in atrial and ventricular 
tissues.

The subcellular fractionation protocol used in the 
present investigation resulted in fractions with relative 
purity at least comparable to those obtained by the tech­
niques of Hui et al. (1976) and of Collins and Cook (1981), 
and it is noteworthy that the procedure does not involve 
the use of high salt extraction results in alterations in 
membrane protein function (See Bers et al., 1979, for 
references). It is evident, through comparison of the 
subcellular fraction marker enzyme specific activity pro­
files with the pr-MDI binding characteristics to the 
fractions, that pr-MDI associates significantly with the 
mitochondrial fraction, which is consistent with the pre­
viously demonstrated direct protective effect of this drug 
on mitochondrial functional integrity (Matlib et al., 
1981). The pr-MDI, however, associates poorly with that 
fraction designated as interface 2, which is highest in 
specific activity for sarcolemmal marker enzyme 
(Na⁺/K⁺-ATPase) at the time (30 minutes) immediately pre­
ceding the maximum peak negative inotropic effect of the 
drugs, and the subcellular distribution of the drug in the 
interface fractions did not correlate with the distribution
of this sarcolemmal enzyme marker, suggesting that under these conditions, pr-MDI is not associating with the sarcolemma. It may be argued that the MDI could have been removed from membrane binding sites during the drug-free washout period following drug perfusion of the hearts. However, this finding of low MDI accumulation in the interface 2 fraction following perfusion of the heart at a concentration of pr-MDI which produced no negative chronotropic effect (thereby excluding calcium entry blockade), coupled with the previous demonstration of an absence of inhibitory effect of this drug on myocardial membrane slow calcium channels or other presumptive membrane calcium channels (discussed in the previous section) in isolated guinea pig atria at the concentration used in the present experiment, provides convincing evidence for a lack of pharmacologically significant sarcolemmal binding of the drug. On the other hand, there is significant accumulation of pr-MDI in the fraction designated as interface 1, which is relatively enriched in sarcoplasmic reticular marker enzyme (NADPH cytochrome c-reductase) activity, and there is a suggestive correlation between the subcellular distribution of pr-MDI in the interface fractions and the fraction profile for the sarcoplasmic reticular marker enzyme. This possible association of pr-MDI with the sarcoplasmic reticulum would be consistent with the inhibitory effect of this drug on calcium mobilization
from this intracellular organelle (Rahwan and Gerald, 1981; Burchfield et al., 1982). It is noteworthy that the high levels of pr-MDI achieved in the mitochondrial and interface 1 fractions by 30 minutes of drug perfusion immediately precede the time of maximum negative inotropic action exerted by this drug in isolated, perfused guinea pig hearts.

The preferential accumulation of pr-MDI in some, rather than all, subcellular fractions following perfusion of the drug into the heart, is taken as evidence of uptake of the MDI into cardiac cells. If the presence of drug in subcellular fractions was an artifact of the homogenization and fractionation procedure, the distribution of drug in all subcellular fractions would have been expected to be homogenous (Rahwan et al., 1973) and not time dependent. Further evidence in favor of uptake of the MDI into cardiac cells derives from the biphasic nature of the washout of the drug following its perfusion into the heart, with the initial rapid phase generally interpreted as representing washout of the drug from the blood vessels and intercellular compartment, while the subsequent slow phase representing the gradual loss of drug from the intracellular compartment (Rahwan et al., 1973; Rubin et al., 1967).
In summary, the present investigation provides evidence for the uptake of pr-MDI into cardiac myofibers, consistent with the assignment of a non-sarcolemmal, intracellular site of negative inotropic action for the tertiary MDI (see discussion in previous section, and Introduction). The pattern of subcellular distribution of pr-MDI correlates with the distribution of mitochondrial and sarcoplasmic reticular marker enzymes but not with the marker enzyme for sarcolemma, in agreement with (and indeed providing a basis of relevance for) pharmacological evidence implicating the mitochondria (Matlib et al., 1981) and sarcoplasmic reticulum (Rahwan and Gerald, 1981; Burchfield et al., 1982) as possible target organelles for the tertiary MDIs, and consistent with the absence of effects of the tertiary MDIs upon sarcolemmal calcium channels at this concentration (as discussed in previous section).
CHAPTER V

SUMMARY AND CONCLUSIONS

1. The tertiary bu- and pr-MDIs have been designated as intracellular calcium antagonists based upon their activities in several in vitro smooth and skeletal muscle systems as well as in the bovine adrenal medullary stimulus-secretion coupling model. Several reports have identified a variety of potential intracellular sites of action for these tertiary MDIs, including sarcoplasmic reticulum, mitochondria, and the calcium binding proteins troponin C and calmodulin. Electrophysiological studies in isolated canine papillary muscle and Purkinje fiber indicate that the tertiary MDIs, as well as the quaternary ammonium Q-bu-MDI, may interfere with both the fast sodium-dependent and slow calcium-dependent components of the cardiac action potential, yet the tertiary MDIs are able to uncouple contraction from excitation in papillary muscle without altering action potential characteristics, supporting a non-plasmalemmal intracellular site of negative inotropic action for the tertiary MDIs.
2. Prior to the commencement of these dissertation studies, information about the effects of the tertiary MDIs on cardiac/cardiovascular systems was limited essentially to the results of an evaluation of the tertiary MDIs in the non-stimulated rabbit Langendorff heart preparation (in which the tertiary MDIs produced a negative inotropic effect and increased coronary flow without altering chronotropy) and in the ouabain-toxic dog (in which the tertiary MDIs were able to both protect against and reverse ouabain-induced arrhythmias). In order to more fully evaluate the cardiac effects of the tertiary MDIs, and to provide information about the putative intracellular site and mechanism of action of the tertiary MDIs, these compounds were evaluated in several in vivo models of experimental arrhythmia with different electrophysiologic and ionic bases of arrhythmogenesis, in in vitro guinea pig spontaneous right and electrically-paced left atrial preparations, and in the in vitro perfused guinea pig heart preparation. A quaternary ammonium Q-bu-MDI, which by virtue of its charge is expected to be excluded from intracellular space, was also evaluated in several of the aforementioned systems for comparative purposes.

3. Pretreatment with both the quaternary and tertiary MDIs afforded significant protection against arrhythmias induced by excess calcium in rats and dogs, and against
arrhythmias induced by chloroform/hypoxia in mice. In both models, calcium ion is thought to play a central role in arrhythmogenesis.

4. The protective activities of the quaternary and tertiary MDIs against arrhythmias induced by aconitine and methacholine in rats were suggestive but not impressive. Alterations in sodium and potassium conductances, respectively, are thought to underly the generation of arrhythmias by these two agents.

5. The ability of the MDIs to provide significant protection against the calcium-dependent arrhythmias, coupled with their unimpressive effects against aconitine- and methacholine-induced arrhythmias, supports the hypothesis that the MDI compounds may interfere in some manner with calcium availability or action.

6. Interestingly, in all models of experimental arrhythmia, the Q-bu-MDI displayed far greater protective activities (on a mg/kg basis) than did the tertiary MDIs or antiarrhythmic standard drugs, including verapamil and quinidine.

7. In an evaluation of the effects of the quaternary and tertiary MDIs upon the mechanical and electrical activities of isolated guinea pig atria, the tertiary MDIs
produced marked negative inotropic effects at concentrations below those at which they produced a mild negative chronotropic effect. Such a profile is consistent with the assumption that calcium entry blockade is not the primary mechanism of action of the tertiary MDIs, since calcium entry blockers produce negative chronotropic and negative inotropic effects in isolated cardiac muscle in identical concentration ranges. Rather, the profile is consistent with a non-plasmalemmmal, intracellular site of negative inotropic action for the tertiary MDIs apparent at lower concentrations. At the higher concentrations, where a slight negative chronotropic effect is observed, the tertiary MDIs may be displaying some membrane activity, including calcium entry blockade, which is indicated by the electrophysiological findings discussed above. The inability of the charged Q-bu-MDI to appreciably alter inotropy further suggests an intracellular site of negative inotropic action for the uncharged tertiary MDIs—-a site from which the Q-bu-MDI is presumably excluded.

8. At very high concentrations (3 x 10^{-4} M), the tertiary MDIs significantly depress frequency-force profile, decrease membrane excitability and decrease following capacity in isolated, stimulated guinea pig left atria. At the same concentration, Q-bu-MDI, which is thought to share most of the electrophysiological properties
possessed by the tertiary MDIs, did not significantly alter these parameters. These findings may indicate that membrane electrophysiological properties alone are not responsible for these effects upon mechanical and electrical activity, or alternatively, may simply reflect a quantitative difference in potency between the tertiary and quaternary MDIs for those electrophysiological properties responsible for the observed effects.

9. In stimulated guinea pig left atria, the negative inotropic effect of the tertiary pr-MDI was reversed by excess calcium, isoproterenol and ouabain, thus satisfying a classical criterion of Fleckenstein (1977) for classification of this MDI as a "calcium antagonist."

10. The characteristics of reversal of the negative inotropic effect of the tertiary pr-MDI by calcium and isoproterenol differed substantially from the reversals of the negative inotropic effects of inhibitors of mitochondrial electron transport and uncouplers of oxidative phosphorylation, indicating that the negative inotropic action of the tertiary MDI is not due to an interference with mitochondrial energy production, as had been suggested by Weishaar et al. (1982).
11. In a pharmacological evaluation of any role of calcium entry blockade in the mechanism of negative inotropic action of the tertiary pr-MDI, it was found that at lower negative inotropic concentrations, pr-MDI did not interfere with concentration-response relationships mediated by calcium and isoproterenol in stimulated left guinea pig atria, indicating that calcium entry blockade is not responsible for the negative inotropic effect of pr-MDI at lower concentrations (≤ 10^{-4} M in guinea pig atria). At higher concentrations (3 x 10^{-4} M), the pr-MDI did antagonize the calcium-mediated concentration-response relationship, indicating calcium entry blockade at these high concentrations. These findings support the existence of an intracellular site of negative inotropic action of the tertiary pr-MDI apparent at lower negative inotropic concentrations, with membrane activity (calcium entry blockade) at high concentrations.

12. Studies were conducted in isolated, perfused guinea pig hearts to determine whether the characteristics of tissue uptake and washout, tissue concentration and subcellular distribution of the tertiary pr-MDI (with 14C-pr-MDI as radiotracer) was consistent with association or availability of the drug to an intracellular site. In these studies, hearts were perfused with 3 x 10^{-5} M pr-MDI (with or without radiotracer). Perfusion with this
concentration of pr-MDI produced a marked negative inotropic effect with relatively no effect upon chronotropy which, based upon the information reviewed above, may have reflected negative inotropic action at an intracellular site.

13. Steady-state uptake of the pr-MDI was achieved at 35-45 minutes of perfusion, at which point the hearts are still extracting 45% of drug in perfusate. Washout indicates an extensive loss of drug from the isolated hearts, with the washout curve being biphasic in nature. The overall characteristics of pr-MDI uptake and washout suggest a ready accessibility of drug into intracellular space.

14. With increasing time of perfusion, pr-MDI accumulated in cardiac tissue (with accumulation in ventricular tissue being greater than in atrial tissue), with high tissue/medium ratios.

15. The time course of negative inotropic effect of pr-MDI paralleled pr-MDI uptake into whole heart and accumulation into atrial and ventricular tissue.

16. A comparison of pr-MDI binding profiles and marker enzyme activity profiles for subcellular fractions of guinea pig ventricle suggest that the tertiary pr-MDI may associate with mitochondria and perhaps with
sarcoplasmic reticulum (previous proposed target organelles for the MDIs), with temporal correlation between accumulation in these fractions and negative inotropic effect.

17. Taken together, the results of the present dissertation provide significant evidence for an intracellular site(s) of action of the calcium antagonistic tertiary MDIs.
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