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Characterization of ATP receptors and voltage-dependent Ca\textsuperscript{2+} channels in cardiovascular cells

Giannattasio, Bartolomeo, Ph.D.
Case Western Reserve University (Health Sciences), 1993
CHARACTERIZATION OF ATP RECEPTORS AND VOLTAGE-DEPENDENT Ca$^{2+}$ CHANNELS IN CARDIOVASCULAR CELLS

by

BARTOLOMEO GIANNATTASIO

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Thesis Adviser: Antonio Scarpa, M.D., Ph.D.

Department of Physiology and Biophysics

CASE WESTERN RESERVE UNIVERSITY

August, 1993
CASE WESTERN RESERVE UNIVERSITY

GRADUATE STUDIES

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Abstract

by

BARTOLOMEO GIANNATTASIO

Chapter 1 is an introduction which describes the molecular mechanisms involved in purinergic transmission in the heart and compares the importance of calcium in the functional regulation of contraction in cardiac and smooth muscle cell.

The following chapters represent the experimental work, including results obtained on cardiac purinergic regulation and inactivation of voltage-dependent calcium channels. Chapter 2 describes the effects of purinergic receptors on the intact atrium and isolated atrial myocytes. This chapter is followed by the molecular characterization of ventricular purinergic receptors by functional expression (chapter 3) and photoaffinity labelling (chapter 4). In chapter 5 data are presented on the inactivation of voltage-dependent calcium channels in the A7r5 vascular smooth muscle cell line.

The primary goal of this study was to characterize myocardial ATP receptors. We used photoaffinity labelling under conditions of high ligand specificity to identify functionally active receptors. Photoaffinity labelling
studies allowed us to characterize two molecules which have been potentially identified as different ATP receptors. The molecular size of the labelled proteins is in the same range as that assumed from the information on the coding gene derived from the functional expression of size fractionated cardiac mRNA. ATP receptors were also functionally expressed by injection of cardiac mRNA in Xenopus laevis oocytes. Cardiac cDNA libraries were made in order to isolate by expression cloning the ATP receptors genomes. This study supports the hypothesis of two different receptor pathways, mediating ATP dependent induction of cation channels or Na+/Pi transport, through the functional expression and the labelling of the two receptors. At this stage the presence of separate receptors also appears from the expression of different pools of library clones. Finally in a separate project we demonstrated that a functionally similar ATP-induced cation conductance in a neuroendocrine cell line is mediated through a larger size message, indicating that ATP-induced cation conductances can be mediated by different molecules. These results have been recently confirmed by labelling experiments in another laboratory (Majiid et al, 1992).

The second part of this study (chapter 5), contributes to the identification of the role of Ca2+ in inactivation of L-type calcium channels. This regulation is relevant not only in defining the gating kinetics of voltage-dependent calcium channels, but also in giving solid basis to models defining the role in vascular smooth muscle of contraction mediated by voltage-dependent calcium channels, as compared to receptor mediated contraction.
This work is dedicated
to Dr. Claudio Sellitti
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CHAPTER 1

CARDIAC PURINERGIC RECEPTORS AND TRANSDUCTION MECHANISMS
A. EXTRACELLULAR PURINERGIC MECHANISMS

ATP has extracellular activities

ATP is an intracellular metabolite, involved in energy metabolism. It is also a substrate for the formation of intracellular mediators (for example cAMP) and intracellular macromolecules (for example DNA). The intracellular concentration of ATP is in the millimolar range and extracellular concentration is normally below micromolar. The extracellular concentration is kept low by the fact that cells are relatively impermeable to ATP and by ecto-nucleotidases which hydrolyze released ATP. Until recently, the major interest has been on the role of ATP in intracellular functions. It is now acknowledged that micromolar extracellular ATP is capable of inducing intracellular signals. These effects are mediated by a variety of plasmamembrane receptors, which belong to different families of extracellular receptors (rev. in Dubyak, 1992).

ATP can be released by different mechanisms

ATP has been shown to be released from cells during pathological conditions, particularly during ischemia (Green and Stoner, 1950). For this reason, the initial studies on extracellular effects of ATP were focused on the cardiovascular system. ATP can also be released as a result of specific signals in many cell types, including blood cells, cardiac myocytes and neurons. The sources can be the cell cytoplasm or specific secretory granules containing ATP (rev. by Dubyak and El-
Moatassin, 1993). The many sources available generate a potential mix of aspecific, pathological, and signal-evoked mechanisms of release. Ubiquitous ecto-ATPases hydrolyze released nucleotides, with enough rapidity to keep extracellular levels below micromolar levels, allowing the possibility for local specific activity (rev. by Dubyak and El-Moatassin, 1993).
B. CARDIAC PURINERGIC MECHANISMS

This part of the chapter is divided in three sections: 1) Overview and history, including classification of purinergic receptors. 2) Mechanisms of ATP release relevant for cardiac purinergic regulation. 3) Cardiac myocardial receptors and transduction mechanisms.

Section 1. Overview and history (classification of purinergic receptors)

Several roles have been proposed for extracellular signaling by ATP. ATP has been considered: an intracellular metabolite which is released by cell lysis and has some local and systemic effects (Green and Stoner, 1950); a neurotransmitter involved in stimulation of autonomic peripheral nerves or of central synapses (Evans et al. 1992, Edwards et al., 1992); hormone with autocrine function (Osipchuk and Cahalan, 1992). This multiplicity is due to the fact that extracellular ATP induces a larger variety of signals than many other extracellular agonists. The variety is a consequence of the many receptors and cell types involved, and the diverse mechanisms of ATP secretion or release (Gordon, 1986, Forrester, 1990, Dubyak and El-Moatassim, 1993).

Evolution of the idea of purinergic signaling

Purinergic receptor-mediated activity on mammalian heart was reported for the first time in 1929 (Drury and Szent-Gyorgyi, 1929). For
many years following this initial report, the major focus of research has been cardiovascular functional regulation by ATP and adenosine. These studies led to the discovery of systemic cardiovascular effects mediated by purinergic compounds. These compounds were thought to be released by pathological cell lysis (rev. by Green and Stoner, 1950). Later, on the basis of the work of Berne (Berne et al., 1963) on the coronary vasodilative effect of adenosine, it was postulated that most of the cardiovascular activity of ATP was mediated by the action of adenosine. Also, it was postulated that purines, released in the blood stream by cells within the heart, could act as local controllers of the coronary circulation (rev. by Burnstock, 1990). Ensuing research demonstrated the presence of electrophysiological effects of purines on the various cardiac functional properties (rev. by Pelleg et al., 1990). Many findings indicate the presence of specific receptors and functions affected by ATP and adenosine, both in cardiac and other cell types (rev. by Burnstock, 1990). The presence of separate ATP and adenosine effects in the heart was first proposed by Burnstock in 1974 (Paddle and Burnstock, 1974).

It has been postulated that ATP might also be considered a neurotransmitter (Burnstock, 1971). This hypothesis originated from the observation of Holton and Holton (1959), who inferred that ATP could be identified as the vasodilative factor released by antidromic stimulation of sensory nerves. The hypothesis was made more credible by Burnstock (rev. by Burnstock, 1969), which demonstrated the presence of a non-
cholinergic, non-adrenergic neural transmission component in autonomic nerves, which was proposed to be mediated by ATP. The idea of purinergic transmission and purinergic nerves was then proposed (Burnstock, 1971, 1972) and expanded by observations indicating the possibility of purinergic control on smooth muscle of several organs (rev. by Burnstock, 1990). The current proposal is that, at autonomic neuromuscular junctions, purines may act as co-transmitters at pre- and post-synaptic junctions (Burnstock, 1990).

The role of ATP as a neurotransmitter is debated, because ATP does not meet unequivocally the classical requirements for a substance to be considered a transmitter (Eccles, 1957, Florey, 1960, McLennan, 1963). This controversy will be described in the following section on the mechanisms of ATP release.

**Classification of purinergic receptors**

Adenosine and ATP act on different types of extracellular receptors. On the basis of the voluminous literature accumulated on the purinergic effects on a variety of tissues, the adenosine P₁ receptors were proposed to be distinct from ATP receptors (P₂) by Burnstock, based on four criteria (Burnstock, 1978): 1) the order of potency of ATP, ADP and adenosine for different effects; 2) the selective antagonism of xanthines on adenosine receptors; 3) the regulation of adenylate cyclase activity by adenosine; 4) the activation of prostaglandin synthesis by ATP. The first two criteria are pharmacological, whereas the last two are functional.
Later (Burnstock and Kennedy, 1985), the P$_2$ ATP receptors were divided into P$_{2x}$ and P$_{2y}$ on the basis of the relative potency of analogues or antagonists (e.g. Hogaboom et al., 1980). The majority of the studies which led to this classification in subtypes was carried out in smooth muscle. According to this classification, the order of potency of ATP analogues on P$_{2x}$ receptors is $\alpha,\beta$-methylene-ATP, $\beta,\gamma$-methylene-ATP and L-$\beta,\gamma$-methylene-ATP > ATP > 2-methylthio-ATP (2-meSATP). $\alpha,\beta$-methylene-ATP in large doses desensitizes this receptor type. For P$_{2y}$ receptors the order is, 2-meSATP > ATP > $\alpha,\beta$-methylene-ATP. Reactive-blue 2 (a compound often only 60% pure) is a relatively selective P$_{2y}$ receptor antagonist. Suramin, a trypanocidal agent is an antagonist of both (Dunn and Blackeley, 1988). This classification was also based on the opposite functional effects on smooth muscle contractility (P$_{2x}$ mediates contraction, while P$_{2y}$ mediates relaxation). The functional discrimination is also supported by the involvement of different transduction mechanisms (P$_{2x}$ receptors induce inward depolarizing currents, and P$_{2y}$ receptors activate G-proteins linked phospholipid cascades). In addition to the presence in smooth muscle, ATP receptors are widely distributed among other cell types, including endothelial cells, where ATP regulates secretion of vasoactive factors (rev. by Gordon, 1986); leukocytes and other blood cells, where ATP controls activation and receptor expression (rev. by Dubyak, 1990, 1992); epithelial cells, where ATP regulates secretion (see in rev. by O’Connor, 1992).
The pharmacological classification of ATP receptors in $P_1$ and $P_2$ has solid basis, since $P_2$ receptors are not activated by adenosine and $P_1$ are not activated by ATP. In addition xanthines are selective antagonist of $P_1$ and not $P_2$ receptors. The functional classification of the same receptors is less straightforward. For example, both ATP and adenosine induce prostaglandin production (see rev. by Engler and Gruber, 1992).

The classification of purinergic receptors into $P_{2x}$ and $P_{2y}$ is insufficient to characterize all the receptor types found in smooth muscle. Many additional receptors are found in other cells. These findings do not contradict the existence of $P_{2x}$ and $P_{2y}$ receptors, but have extended the number of potential receptor types. The review of Gordon, 1986, includes: $P_{2x}$, $P_{2y}$, $P_{2t}$ (a receptor in platelets selective to ADP), $P_{2z}$ (a receptor demonstrated in macrophages, mast cells and fibroblast cell lines, selective to the anionic form of ATP and forming large non-selective pores). In addition, there are receptors in leukocytes, smooth muscle and endothelial cells that are activated by ATP as well as by UTP. These receptors include more than one type, and have been defined as $P_{2u}$ (Dubyak, 1990), or "nucleotide" receptors (rev. in O'Connor, 1992), since are equally affected by purines and pyrimidines.

*Problems in ATP receptor classification*

The classification of ATP receptors is hindered by the presence in the same cell type of more than one class of receptors and the possibility that some of the ATP analogues can be degraded to adenosine, which
has its own receptor-mediated activity. Both factors may contribute to make questionable the order of potency of analogues on a single receptor and, perhaps, to assume the presence of an unrealistic number of different receptors. These problems have induced many to consider an operational definition on specific transduction mechanisms as the criterion for receptors differentiation or classification. This criterion might utilize physiologically relevant differences between receptors.

The cloning of the receptors gene would permit a better classification, making it dependent on similarities in the protein primary sequence. Even the slight pharmacological difference among ATP analogues could be based on a large structural difference between receptor types. It is possible that there could be many primary sequences of ATP receptors, leaving still open the necessity of knowing which receptors are expressed in different cell types. This knowledge is practically relevant, for example to design a drug selective for the regulation of a specific function. On the other hand the larger number of ATP receptors and cell types involved in purinergic mechanisms as compared to other mediators could undermine the success in designing specific drugs.

The presence of extracellular ATP receptors also stretches the definition of receptors as separate molecules from the others, since ATP form complexes with many molecules, even at the plasmamembrane. Hence the differentiation from membrane ATPases, kinases, etc. rests on
the affinity of the complex formation (ATP receptors bind ATP at micromolar level) and the generation of specific cell signals.

Section 2. Possible sources of extracellular ATP in the heart

ATP is widely present in cells and organelles, including secretory granules (rev. in White and McDonald, 1990, Inoue, 1992). Consequently, ATP can be released as a result of cell lysis or specific secretion by many cell types including myocardial cells, blood cells and neurons (rev. by Gordon, 1986, Pelleg et al., 1990, White and McDonald, 1990, Forrester, 1990, Dubyak and El Moatassim, 1993). The many sources of ATP release create a potential mix of unregulated and specific release, both under physiological and pathological conditions. This mix increases the possibility of functional regulation by purinergic mechanisms, but also decreases their specificity and hinders the understanding of the relevancy of ATP effects.

Potential modalities of ATP release include: 1) physiological and pathological release from myocardial cells; 2) physiological and pathological secretion by blood cells; 3) neurotransmission.

The mechanisms of autocrine release from myocardial and blood cells are described in many reviews (e.g., Gordon, 1986, Forrester, 1990, Dubyak and El Moatassim, 1993). Nanomolar levels of ATP and adenosine are found under physiological conditions in the venous coronary flow. The amount of ATP found in these venous effluents may increase with increase in cardiac work. In ischemia and hypoxia, ATP
released in the coronary flow is increased. This increase precedes release of cellular enzymes or other signs of cell damage (Green and Stoner, 1950, Paddle and Burnstock, 1974, Forrester, 1990). In both physiological and pathological conditions the concentration of ATP measured in the coronary venous effluent might be lower than the concentration at the site of release, due to rapid and elevated extracellular ecto-nucleotidase activity which has been demonstrated in the heart (rev. by Forrester, 1990). Local concentration can be increased in ischemia and other pathological conditions by blood cells involved in the pathological process or by myocardial cell exocytosis or lysis. Because ATP receptors of cardiac and blood cells respond to low micromolar concentration, a role of purines both in physiological and pathological cardiac functional control can be visualized.

The mechanisms of release described above are local autocrine processes. Systemic release of ATP is also possible, for example in case of massive trauma (Green and Stoner, 1950). Potentially, also neural ATP release is relevant to the ATP cardiac regulation (Burnstock, 1990).

The many sources from which ATP could be released generate a possible mixture of neural and autocrine signals. The signals evoked upon purinergic receptor activation may modulate the responses to neurotransmitters, such as acetylcholine or norepinephrine, released from cardiac nerve terminals.

Most of the evidence on ATP neural release indicates its potential role as a co-transmitter (see below). There is also histochemical and
electrophysiological evidence indicating the presence of a non-cholinergic, non-adrenergic transmission in the vagal nerves of the heart (Crowe and Burnstock, 1982, Weihe, Reinecke and Forssmann, 1984, Konishi Okamoto and Otsuka, 1985, Konopta McKeon and Parson, 1989). It has been proposed that this neural component is (in part) purinergic (Crowe and Burnstock, 1982, Fieber and Adams, 1991).

**ATP may be a co-transmitter**

Co-transmission is the secretion by the same neuron of more than one transmitter. Experimental evidence suggests that ATP might act as a co-transmitter at the junction between autonomic neurons and cardiac cells (Donald, 1985, Hoyle and Burnstock, 1986, Burnstock, 1990). ATP is co-stored in synaptic vesicles with norepinephrine and acetylcholine (Silinski, 1975, rev. by Westfall et al., 1990). ATP is also released together with catecholamines in sympathetic nerves and with acetylcholine in cholinergic nerves (rev. by Stone, 1981, White and McDonald, 1990).

The classical requirements for a substance to be considered a transmitter include (Eccles, 1957, Florey, 1960, McLennan, 1963): 1) storage of the substance in pre-synaptic terminations and mechanisms able for its synthesis; 2) release upon stimulation; 3) presence of post-synaptic receptors responsible for the observed function; 4) similarity in the effect generated by neural stimulation and application of the chemical; 5) presence of mechanisms of uptake, synthesis, or degradation of the substance. These criteria have been designed for the release of one
compound at any neural termination. In the case of ATP signaling, the criteria are difficult to evaluate because ATP may be released by neural and non-neural mechanisms. Nonetheless, fulfillment of these criteria could help the assessment of the role of ATP in neural regulation of cardiac function.

The criteria of storage and release in cardiac nerves (Silinski, 1975, Ishizuka et al., 1978, Hartzell, 1979, Stone, 1981, Burnstock, 1981, Crowe and Burnstock, 1982) and in nerves of other tissue (rev. by White and McDonald, 1990, Wetfall et al., 1990) are met by ATP. The presence of receptors on cardiac cells (third criterion) is also met. This evidence is presented in the following section of this study. Membrane ecto-nucleotidases (fifth criterion) have been described in many cell types (rev in Slakey et al., 1990, including heart (Naito, Y. and Lowenstein, J.M., 1985). The fourth criterion is critical since it defines a role for ATP-mediated neural stimulation, as compared to the release of ATP by cell lysis or by cells other than neurons. There is evidence for ATP mediated neural effect in heart, based upon ATP-like effects generated by neural stimulation and block by ATP antagonists (Hartzell, 1979, Donald, 1985, Hoyle and Burnstock, 1986, Pelleg and Michelson, 1987, Pelleg et al., 1985, 1987, 1988), and in other tissues (rev. by Burnstock, 1990, White and McDonald, 1990, Westfall, 1990, Benham, 1992). This evidence is weakened by the undemonstrated specificity of available antagonists. In the work of Edward et al., 1992, and Evans et al., 1992, suramin seems adequate to demonstrate this specific criterion in peripheral and central
neural synapses. Using this antagonist to study neural stimulation of cardiac function, however, is limited by the presence of many ATP receptors in the various tissues present in the heart.

The presence of many ATP receptors and the absence of specific antagonists are the rationale for the experiments presented in following chapters on the characterization of the ventricular ATP receptors by expression cloning and labeling. The above indicated hindrances also increase the usefulness of the identification of 8-azido-ATP as a potential selective antagonist of ventricular purinergic receptors and the utilization of this compound for labelling of these receptors (chapter 4 of this study).

In conclusion, a large body of evidence support the role of neural release of ATP in cardiac neural regulation. However, this evidence is weakened because of the following specific issues: 1) the absence of specific antagonists for ATP receptors; 2) the presence of many molecules binding ATP even at the membrane level; 3) the fact that none of these receptors has been isolated; 4) the presence of a potential mixture of specific and aspecific mechanisms of release. The fact that ATP may be released by the non-neural mechanisms described in the previous paragraph separates ATP from other classical transmitters. The determination of neural release of ATP could be nonetheless relevant, since ATP has effects on the heart and modulates effects of other extracellular signals (see below).

*Co-transmission with acetylcholine*
The presence of combined effect and co-release of ATP and acetylcholine has been indicated in different tissues (rev. by Burnstock, 1985). In the heart, but not in other tissues, some researchers have proposed that in presence of acetylcholine, ATP would mostly act through hydrolysis to adenosine (Pappano and Mobagwa, 1992). This tissue variability depends on the receptor types present and the different transduction mechanisms involved. For example in skeletal muscle the prevalent $P_{2X}$ like receptors (Hume and Honig, 1986), mediate ATP potentiation of inotropism.

In some cases it is not clear if the effect of ATP and that of acetylcholine are separated. For example it has been reported that ATP in bullfrog sympathetic neurons increases sensitivity of the nicotinic receptor for cholinergic agonists (Akasu and Koketsu, 1985). These authors suggest allosteric binding of ATP to the nicotinic receptor. In Xenopus muscle (Igusa, 1988), ATP may activate directly the nicotinic receptor. In other cell types including parasympathetic cardiac ganglia neurons (Fieber and Adams, 1991) this finding has not been confirmed. In some cases nicotinic receptors and $P_{2X}$ receptors are both present. In the study of Fieber and Adams, the distinction between the ATP activated conductance and the nicotinic receptor is based on at least two types of evidence: 1) different reversal potentials of the respectively activated currents; 2) absence of cross-desensitization and presence of separate antagonists.
**Pre-junctional effects:**

Most experimental work suggests that in the autonomic nervous system P₂ receptors are located on the post-junctional side of neuro-effector junctions (Burnstock, 1981). Released ATP can be metabolized to adenosine by ecto-nucleotidases, at a rate which depends on the tissue (Burger and Lowenstein, 1970, Harris, 1972, Nagy et al., 1983 and Pearson et al., 1985). Previous work has indicated that while P₂ receptors act post-junctionally, adenosine receptors (P₁) act pre-junctionally (De Mey, Burnstock and Vanhoutte, 1979). Stimulation of pre-junctional P₁ receptors has been shown to reduce acetylcholine release at several neuro-muscle junctions (Ginsborg and Hirst, 1972, Ribeiro and Walker, 1973, Vizi and Knoll, 1976 Gustaffson et al., 1978, Moody and Burnstock, 1982, Reese and Cooper, 1982, Somogyi and Vizi, 1987). Norepinephrine release can similarly be decreased by ATP and adenosine in a variety of tissues (rev. in Hedquist and Fredholm, 1976) including vascular preparations, (rev. in Su, 1978). Adenosine effects are mediated by A₁ receptors, (Paton, 1981). More recent evidence indicates that P₂x receptors can also increase secretion of neuromediators at the terminal of autonomic nerves (rev. by Silinsky et al., 1990, Sperlagh and Vizi, 1991). In the latter study P₂x and P₁ receptors were both present and elicited opposite responses. In other studies it has been proven that P₂y receptors may inhibit neuromediator release pre-synaptically (Shinozuka et al. 1988, Forsyth et al. 1991, Goncalves and Guimaraes, 1991). A more complete picture of pre-synaptic control should include, together with the
other mediators and adenosine control, ATP regulation through stimulatory or inhibitory receptors (rev. by Silinski et al. 1990).

*ATP regulation of cardiac parasympathetic neurons*

Purinergic regulation of the cardiac function could be relevant at the level of the parasympathetic neurons present in the sub-epicardium. These neurons are part of the cardiac vagal transmission. Histochemical and electrophysiological evidence indicates a non-cholinergic, non-adrenergic transmission component, part of the vagal nerves of the heart (Crowe and Burnstock, 1982, Weihe, Reinecke and Forsmann, 1984, Konishi Okamoto and Otsuka, 1985, Konopta McKeon and Parson, 1989). This component has been proposed to be purinergic (Crowe and Burnstock, 1982). ATP receptors and ATP mediated transduction effects are present in 75% of isolated sub-epicardial parasympathetic neurons of mammalian ganglia. In these cells ATP induces cationic currents (Fieber and Adams, 1991) with an high $\text{Ca}^{2+}/\text{Na}^+$ permeability ratio. Cationic currents of parasympathetic cardiac neurons, as the ones of cardiac ventricular cells (Zheng et al., 1992), are activated by purinergic receptors similar to the $P_{2\gamma}$ type (they are activated by 2-methylthio-ATP and not by $\alpha,\beta$-methylene-ATP or $\beta,\gamma$-methylene-ATP). This is different from the ATP activated cationic conductance in most other cell types, where the ATP receptor pharmacology identifies the receptors as $P_{2\alpha}$ type (Bean, 1992). However, the activation of these $P_{2\gamma}$ receptors is extremely fast. Activation time constant is about 20 ms in the parasympathetic ganglia.
and about 100 ms (which was the limit of the solution changing apparatus used), for the cardiac conductance (Zheng, Ph.D thesis, 1992). These activation time courses indicate a direct coupling of receptors to the cation channels. This fast time course is more typical of $P_{2\delta}$ activated conductances (Bean, 1992).

Slightly different conductances are activated in other neurons, like frog sensory neurons (Bean, Williams and Ceelen, 1990), rat sensory neurons (Krishtal et al., 1983, 1988), dorsal root ganglia (Bean, 1990). These receptors are of the $P_{2\delta}$ type. Different receptor types are present in PC12 neuroendocrine cells, where ATP affects secretion of norepinephrine (Inoue and Nakazawa, 1992).

**Conclusion:**

In conclusion a large body of evidence has been accumulating on the role of ATP as a co-transmitter at the sympathetic and parasympathetic neuro-effector junctions of many visceral and vascular smooth muscles, and other autonomic innervation, including the heart. ATP can be systemically released in pathological conditions (rev. by Green and Stoner, 1950), or locally by myocardial cells under physiological or pathological conditions (rev. by Forrester, 1990). ATP may also be released by blood cells (rev. by Gordon, 1986, Dubyak and El Moatassin, 1993). In leukocytes ATP may have a role as an autocrine mediator of cell to cell communication (Osipchuk and Cahalan, 1992). Recent studies also indicate that the "multidrug transporter", a large anion
transporter present in several cell types, may release ATP (Dr. Guidotti, personal communication). Finally, ATP may regulate gap-junctions in the heart (Sugiura et al., 1992) and other tissues (Henkvist and McCarthy, 1992). Hence, ATP can participate in many types of signaling. A complete description of purinergic cardiac functional regulation will have to consider these signals and the many receptors able to initiate them.

Section 3. Cardiac Purinergic receptors and transduction mechanisms

Potential sites of activity

In heart, purines may act on different cell types. Only 40% of the cells in the heart are ventricular myocytes. Other cell types can be involved in purinergic regulation through specific receptors. The cell types that can be relevant to purinergic transmission include: nodal and conduction cells, atrial and ventricular myocytes, endothelial cells, and mesenchymal structures, including the specific cardiac circulation. Nodal cells and conduction fibers have a pace-maker function for the origin of the electrical wave and a propagation function which ensures coordinated diffusion of electrical depolarization. Myocardial cells contract upon depolarization and are responsible for the generation of the cardiac beat. Hence, nodal and conduction fibers are the cells responsible for pacing properties, while myocardial cells are directly responsible for inotropism and only indirectly participate in pacing. Functional control of the heart is
also determined through control of the cardiac coronary circulation. The coronary circulation is a low-resistance terminal circulation. The low resistance is necessary since the only possibility for blood to flow through the muscle is during diastole. In the coronary regulation, purinergic and other transmitters have to ensure a low resistance pathway under any condition.

The extracellular effects of purines on all these cell types are mediated through adenosine and ATP receptors. The effects produced on nodal and atrial cell receptors are relevant for functional studies on intact atrium, hence some information on these receptors is given in the following chapter. In this chapter, previous work will be reviewed on the mechanisms and receptors involved in purinergic activity on ventricular myocytes, since ventricular myocytes receptors are the principal object of this study.

*ATP dependent transduction mechanisms in ventricular myocytes*

Extracellular ATP has positive inotropic effects on ventricular cells or intact heart, when added together with norepinephrine (Danzinger et al., 1988, Legssyer et al., 1988, Scamps et al., 1990). The potentiating effect of norepinephrine is mediated by beta 1 receptors (Scamps et al., 1990 and this study).

Studies in our laboratory and others have shown that in ventricular myocytes, micromolar extracellular ATP increases intracellular calcium (De Young and Scarpa, 1987, Christie et al., 1992, Danzinger et al., 1988).
The ATP induced calcium transient is increased if norepinephrine was previously added (De Young and Scarpa, 1987). This calcium signal could regulate the contractile function of ventricular myocytes. ATP effects on calcium homeostasis in myocytes are mediated by several receptor types and transduction mechanisms. For this reason, the mechanisms involved in calcium regulation and contraction of ventricular myocytes are summarized below.

*Excitation-contraction coupling in ventricular cells*

In ventricular myocytes, action potentials initiated through sodium channels, or other types of depolarization, activate voltage-sensitive calcium channels that participate to the cardiac action potential. This activity generates \( \text{Ca}^{2+} \) influx. Calcium can activate \( \text{Ca}^{2+} \) release from intracellular stores through receptors which bind ryanodine, and are activated by calcium increases. The cardiac sarcoplasmic reticulum (SR) is the principal intracellular store of calcium, and contains both ryanodine receptors and 1,4,5-inositol-phosphate receptors (IP\(_3\)-receptors) (Kijima et al., 1993). Both are ligand-operated \( \text{Ca}^{2+} \)-release channels which belong to a similar family of molecules, since they have conserved sequences, are characterized by large size, and are homopolymers of four molecules. Ryanodine receptors serve as the \( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \)-release channels and are considered functionally similar to the spanning region that mediates direct coupling between membrane depolarization and calcium release in skeletal muscle cells (rev. by Huang, 1988). The
role of IP3-receptors in heart is less defined. Recently Kijima et al., 1993, demonstrated that ryanodine- and IP3-receptors are localized in different regions in myocardial cells. Ryanodine receptors are localized at the junctional SR and IP3-receptors at the region of intercalated discs both in atrial and ventricular myocytes. This different localization might correlate with the regulation of different functions.

In conclusion, depolarization and calcium influx through voltage-dependent calcium channels in cardiac myocytes can be associated with release of calcium through different mechanisms, including intracellular Ca\(^{2+}\) release dependent on Ca\(^{2+}\) influx. In addition, in myocardial cells calcium is the principal regulator of contraction (rev. by Sperelakis and Banks, 1993). For these reasons, in myocytes depolarization and calcium influx are sufficient to generate maximal contraction. This is different than smooth muscle cells (see following section).

In myocytes, ATP operates the only receptor-operated calcium influx so far identified in these cells. The ATP activity controls both depolarization and calcium flux (see below).

**ATP dependent Calcium transients in ventricular myocytes**

De Young and Scarpa 1987, 1989 and 1991, have shown that in myocardial cells micromolar concentrations of extracellular ATP generate a rapid (about 1 min) transient in intracellular calcium. This effect is dependent on the presence of extracellular Mg\(^{2+}\). UTP, \(\alpha,\beta\)-methylene-ATP, \(\beta,\gamma\)-methylene-ATP and adenosine do not mediate a similar effect.
ATP₇S induces a slower monophasic increase in calcium which is not preceded by the rapid transient (De Young and Scarpa, 1987, 1989, 1991, Christie et al., 1992). 2-methylthio-ATP generates a rapid transient of calcium which returns to baseline conditions, in contrast to the effect of ATP₇S (De Young and Scarpa, 1991). Danzinger et al., 1988, have demonstrated that influx of extracellular calcium is required for the ATP induced transient. De Young and Scarpa, 1989, have demonstrated that intracellular release and influx of calcium are both part of the effect induced by ATP. In the latter experiments calcium transients can be eliminated by chelating extracellular calcium with EGTA. Calcium channel blockers significantly decrease, but do not suppress, ATP induced calcium transients. Depletion of intracellular ryanodine-sensitive Ca²⁺ stores also decreases calcium transients. Similar results were found by Christie et al., 1992. These results demonstrate that ATP induces: 1) Ca²⁺ influx unrelated to voltage dependent L-type calcium channels. 2) Ca²⁺ influx through L-type calcium channels. 3) Ca²⁺ release from ryanodine-sensitive stores.

In subsequent studies, it has been shown that in ventricular myocytes extracellular ATP activates a non-selective cation current (Christie, 1990, 1992, Zheng et al., 1992). This current is similar to the one activated by ATP in atrial myocytes (Friel and Bean, 1987). Single channels activated by ATP in ventricular myocytes have very low permeability, about 1 pS (Zheng, Ph.D. thesis 1992), as compared to ATP activated channels in other excitable cells (rev. by Bean, 1992).
Christie et al., 1992, have shown that the ATP activated current can depolarize isolated ventricular myocytes 15-20 mV. The depolarization is able to induce action potential mediated by voltage-dependent sodium and calcium currents. Depolarization is still present in absence of calcium in the extracellular medium or in presence of block of voltage-dependent sodium channels by tetrodotoxin (TTX) and L-type calcium channels by dihydropyridines (DHP). However these conditions suppress action potentials. Although the ATP induced depolarization is not blocked by TTX or DHP antagonists, it is blocked by quinidine (Christie et al., 1992). This latter is an interesting finding since this compound is used as an antiarrhythmic. A model representing these events is shown in the figure (left model).

The results shown above indicate that extracellular ATP$_7$S induces a slow monophasic increase in cytosolic calcium, 2-methylthio-ATP induces a rapid transient and ATP induces the rapid transient followed by a slow increase. This observation was followed by additional experiments (De Young and Scarpa, 1991) which have shown that the delayed phase of the ATP response requires inorganic phosphate in the extracellular buffer. In zero phosphate, the changes in intracellular free Ca$^{2+}$ induced by 2-methylthio-ATP and ATP are similar. Under the same conditions ATP$_7$S has little or no effect. As the extracellular Pi is increased from zero to 10 mM, a parallel increase in the ATPcS effect is observed. The delayed slow phase of intracellular Ca$^{2+}$ increase induced by ATP is similarly dependent on phosphate concentrations. Extracellular sodium is
also necessary for observing the response in the presence of Pi. Christie et al., 1992 had proposed that the different time course between the effects of ATP and ATP\textsubscript{γ}S was due to the different kinetics of a putative phosphorylation process induced by the binding of either nucleotide to the same receptor. According to this model phosphorylation was necessary for the activation of the ATP induced cation conductance. By contrast, the data reported above are more consistent with the presence of two separate receptors mediating the 2-methylthio-ATP and ATP\textsubscript{γ}S effects (De Young and Scarpa, 1991). Binding of 2-methylthio-ATP or ATP to one of these receptors activates the cation conductance. ATP\textsubscript{γ}S or ATP induces activation of a Na\textsuperscript{+}/Pi transporter by binding to the other receptor. The Na\textsuperscript{+}/Pi transporter has been shown in several cells including cardiac myocytes, (Jack et al., 1989). Thus, the slow transient of calcium induced by ATP\textsubscript{γ}S or ATP is consistent with an activation of sodium transport into the cells, followed by an exchange with extracellular calcium by the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger.

Voltage-clamp experiments in isolated ventricular myocytes have demonstrated that activation of the cation current induced by ATP or 2-methylthio-ATP is not mediated by a G-protein. The measured time constant of this activation is about 100 ms (Zheng et al. 1992, Zheng, Ph.D thesis, 1992). This value may be an overestimate since it is close to the mixing time of the apparatus used for changing extracellular solution. That evidence is compatible with the hypothesis that binding of ATP and
2-methylthio-ATP to this receptor induces ligand-operated direct activation of the cationic current.

ATP and 2-methylthio-ATP induce also an increase in calcium influx through L-type calcium channels. Preliminary evidences indicate that a pertussis toxin sensitive G-protein is involved in this receptor-operated process (Zheng et al., 1992). This transduction mechanism appears to be mediated by different receptors and is distinct from the ATP dependent activation of the cation current. In fact, the ATP induced activation of L-type channels has a half time of seconds, involves G-proteins and is dependent on the Mg$^{2+}$-free form of ATP (Zheng et al., 1992). Also, in continuous presence of the agonist, the ATP-activated cation conductance desensitizes in few seconds, while the activation of L-type channel is a prolonged effect.

Other evidences by Scamps et al., 1992 indicated that the activation of L-type calcium channels induced by ATP in rat ventricular myocytes is mediated by a cholera toxin sensitive G-protein, not coupled to adenylate cyclase. However, in ferret myocytes, ATP inhibits L-types calcium channel activity, possibly through P2y receptors (Qu et al., 1991). These receptors are linked to phospholipase C activation and inositol-triphosphate production. The data infer that both ATP dependent activation and inhibition of L-type channels are possible. These effects might be mediated by G-proteins, potentially through different mechanisms.
Both the works of Scamps et al., 1992 and Zheng et al. 1992, indicate that ATP dependent activation of L-type calcium channels and of the cation conductance involve separate receptors. However according to Scamps and Vassort, 1990, Vassort et al., 1992, Mg$^{2+}$-ATP does not directly activate a cation channel, but a HCO$_3$/Cl exchanger which generates acidification. In their model acidification induces a Ca$^{2+}$ transient mediated through displacement of calcium by protons. The calcium transient activates a cation conductance. On the basis of the time course of activation of the ATP induced cation conductance our laboratory prefers the mechanism involving direct activation.

Pucaet et al., 1993, proposed that ATP as well as alpha adrenergic stimulation also activate a Na$^+$/H$^+$ exchanger. It has been proposed that this effect is mediated by G-protein linked P$_{2y}$ receptors, even though it is independent on protein kinase C activation (Pucaet et al., 1993).

Extracellular ATP also increases the production of inositol-triphosphate and diacylglycerol (Leggsey et al., 1988) in rat, rabbit (Takikawa et al., 1990) and mouse myocytes (Yamada et al., 1992). These increases are mediated by phospholipase C activation, and occur through the activation of P$_{2y}$ receptors. In these experiments activation by ATP decreases the intracellular concentration of cAMP. The relevance of the phospholipase C cascade in the regulation of cardiac calcium and contraction is not clear. From previous evidence, its participation in calcium transients in rat ventricular myocytes appears to be minimal (De Young and Scarpa, 1989, Zheng et al., 1992). However, in cells where
this mechanism is present, it might inhibit the ATP dependent mechanisms activating calcium influx. In fact, a decrease in cAMP may inhibit the component of calcium influx dependent on L-type calcium current activation (see below).

Finally, it has been reported in myocardial cells (Sujira et al., 1991) as well as in astrocyte (Enkvist and McCarthy, 1992), that ATP modulates gap junction conductances.

_modulation of ATP effect by norepinephrine_

De Young and Scarpa, 1987, 1989, have demonstrated that the pretreatment of myocytes with norepinephrine for 30-60 sec increases the ATP-induced Ca\(^{2+}\) transient. This potentiation is not affected by previous depletion of ryanodine sensitive stores. This effect, which is mediated by activation of adenylate cyclase and protein kinase A (Zheng et al. 1992), involves increased flow of calcium through L-types channels (Warbanow and Wollemberg, 1982, Reuter, 1983, Trautwein and Hescheler, 1990). Norepinephrine itself does not increase the ATP dependent cation conductance (Zheng et al., 1992).

Zheng et al. 1992, have shown that protein kinase C activation by phorbol esters reduces the ATP dependent increase in calcium. This effect is mediated by reduction of calcium influx through L-type channels. The ATP induced cationic current is not modified by protein kinase C activity.
Protein kinase C may be activated by receptors that also induce intracellular calcium release. Interestingly, the ATP response is also potentiated by addition of cis-polyenic fatty acids (FA) which mobilize intracellular $\text{Ca}^{2+}$ release (Damron and Bond, 1993). FA can be released through phospholipase $A_2$ or phospholipase $C$ activation and diacylglycerol release. FA inhibit electrically stimulated $\text{Ca}^{2+}$ transients, but increase ATP transients.

The two mechanisms of potentiation of the ATP response by norepinephrine and FA probably involve "cross talk" regulation. In myocardial cells these mechanisms might regulate ATP dependent calcium increase by influx or intracellular release.

In conclusion, extracellular ATP activates many intracellular signals in ventricular myocytes. In spite of recent progress describing the various responses, there is a need for a more clear definition of the receptors present and their relevance to cardiac functional control. The goal of this study was to identify receptor molecules and relevant cardiac genes involved.
C. RELEVANCE OF ATP RECEPTORS AND L-TYPE CALCIUM CHANNELS IN CALCIUM SIGNALS AND CONTRACTION IN CARDIAC AND VASCULAR MUSCLE

In cardiac and vascular smooth muscle cells, intracellular calcium is an important transducer between plasma membrane excitation and contraction. In skeletal, myocardial, and smooth muscles excitation-contraction coupling involves different molecular mechanisms. In all three types of muscle, the increase in intracellular calcium is a common determinant factor in contraction, but this increase is determined by different molecular mechanisms. Three major mechanisms of excitation-contraction coupling have been described (rev. by Sperelakis and Banks, 1993): 1) direct coupling between membrane voltage sensors (dihydropyridine receptors, DHP-R) and release of calcium from sarcoplasmic reticulum (rev. by Huang, 1988); 2) activation of inositol-triphosphate production, via phospholipase cascade, with subsequent intracellular calcium release; 3) release of calcium induced by calcium influx (Ca\(^{2+}\)-mediated Ca\(^{2+}\)-release). One of these mechanisms is predominant in any muscle cell type. The calcium transient in cardiac cells depends on influx of extracellular calcium, and Ca\(^{2+}\)-induced Ca\(^{2+}\)-release.

Ventricular myocytes are somewhat more differentiated than smooth muscle, consistent with their inferior potential for division and higher specialization. The specificity of this role may be based on fewer
regulatory factors. In cardiac myocytes, calcium is the most important effector of contraction. In addition, it participates in action potentials, automaticity, and the induction of its own release from the sarcoplasmic reticulum. Finally, calcium regulates contractile force by binding to intracellular proteins which are involved in regulation of contraction (rev. by Sperelakis and Banks, 1993).

In cardiac cells, electrical stimulation of contraction is more important than receptor-mediated (pharmaco-mechanical) coupling. However extracellular ATP, endothelin, neuropeptides, norepinephrine and more recently cis-polyenic fatty acid (FA) (Damron and Bond, 1993), are involved to various extent in regulation of ventricular functions. Of these, only ATP and FA directly increase intracellular calcium. An ATP agonistic activity on myocytes was proposed (Paddle and Burnstock, 1974) and then characterized in terms of transduction and receptors involvement (De Young, 1989, 1991, Zheng, 1992, Christie, 1991, 1992, and this study).

In smooth muscle, hormone- or other signal-mediated contraction is more significant than in cardiac cells (Nelson, 1990). This is possibly due to the larger variety of contraction modes present in these cells compared to cardiac cells. For example, unlike myocardial cells, smooth muscle cells can tonically contract. For the same reason, in these cells phosphorylation of contractile and regulative proteins and other biochemical intracellular signals are more critical for the regulation of contraction (rev. by Somlyo and Somlyo, 1992). Hence, in these cells
contractility is regulated by calcium-dependent and independent intracellular signals. Extra- and intracellular signals involved in contraction regulation vary in smooth muscle of different organs and tissues (rev. by Nelson, 1990, Somlyo and Somlyo, 1992). For example the participation of ATP and other extracellular agonists in functional regulation of vascular smooth muscle cells varies among vessels and vessel size. As the ramification from central to peripheral vessels progresses, receptor-mediated contraction becomes more dependent on voltage-dependent calcium channels (Van Breemen et al. 1989, see also Nelson et al., 1990). As the consequence of a variable cell differentiation, sections of the vascular tree may differ in sensitivity to purinergic mediators or other extracellular regulatory factors. A further source of variability in the contractile regulation by extracellular signals is due to physiological changes in the differentiation status of these cells. For example, in the uterus there are pregnancy-induced changes of the dependency of smooth muscle contraction, from the very active cholinergic control to the less active purinergic regulation that prevails during the later phases of the pregnancy (Levin et al., 1991). These findings suggest also hormonal regulation of the expression of purinergic receptors.

In cardiac myocytes and in vascular smooth muscle, extracellular signals induce effects on contraction through depolarization and membrane receptors. As discussed previously, purinergic transmission acts in the heart primarily via depolarization and influx of calcium, through activation of purinergic-induced cation conductance and voltage-
dependent calcium channels. In smooth muscle, voltage-dependent calcium channels, action potentials and the activity of chemical transmitters are all involved in contraction (rev. by Nelson, 1990 and Somlyo and Somlyo, 1992). Models have been presented on the relative participation of receptor-operated and voltage-dependent channels in vascular smooth muscle. Nelson et al., 1990, presented a model in which regulation of tone in middle size arteries was due mostly to small changes of voltage, which regulate calcium primarily through voltage-dependent calcium channels, even in the absence of action potentials. According to Nelson’s model, many vasoconstrictors work through small depolarization. This depolarization generates sufficient Ca^{2+} influx to induce contraction. In the same model many vasodilators work through activation of a potassium current regulated by intracellular ATP (K_{ATP}). It is known however that some vasoconstrictors can act in absence of depolarization and other smooth muscle cells can develop action potentials (Somlyo and Somlyo, 1992). In conclusion, the effect of calcium in the regulation of tone in smooth muscle will depend on the presence of other intracellular signals (Somlyo and Somlyo, 1992). For example, in vascular smooth muscle, P_{2X} receptors induce contraction through depolarization and calcium influx while P_{2Y} receptors generate increase in calcium transient and relaxation.

Different smooth muscles have dissimilar mechanisms of excitation-contraction coupling. The role of pharmaco-dependent and voltage-dependent channels in the excitation-contraction coupling of
these muscles may be evaluated by determining the receptors present and the regulation of the voltage-dependent channels which participate to the influx of calcium. In the case of voltage-dependent calcium channels quantitative kinetic models might help to define the role of these molecules in more complex functions (like action potentials or influx of calcium and excitation-contraction coupling). The data presented in chapter 5 on the inactivation of the voltage dependent calcium channels, in a vascular smooth muscle cell line, could be used for such integration. This kinetic model is currently being used in our laboratory (Dr. C. Obejero-Paz personal communication) as part of a simulation of the action potential in the smooth muscle cell line A7r5.
Figure 1. A model representing ATP regulation of calcium channels in ventricular cells. Top left side shows activation of ATP dependent cation influx and voltage dependent calcium channels. Top right side shows ATP dependent activation of Na/Pi transport and Na/Ca exchange. The nature of the receptor coupling to these mechanisms is not known. Bottom figure shows calcium induced calcium release from the sarcoplasmic reticulum, which can be activated by the calcium influx.
ATP Receptor Modulation of Cell Calcium

Figure 1
CHAPTER 2

EFFECTS OF ATP AND ADENOSINE ON INTACT ATRIUM AND ISOLATED ATRIAL MYOCYTES.
ABSTRACT

1. Background: Systemic regulatory function of purinergic compounds on intact heart and atrium.

2. Experimental data:

   I. ATP and adenosine effects on isometric tension in the resected and spontaneously beating atrium. The data show that purinergic control of tension and pacing is mostly mediated through adenosine, with additional specific ATP effects. ATP, at variance from adenosine, may potentiate atrial contraction, when the atrium is pre-exposed to norepinephrine (NE). The NE effect is mediated through beta 1 activity.

   II. Preparation of isolated functional atrial myocytes.

   III. Evidence that in isolated atrial myocytes extracellular ATP, but not adenosine, is able to generate a calcium transient similar to the one activated in ventricular cells. Pretreatment with norepinephrine markedly increases the transients.
INTRODUCTION

A. RATIONALE FOR STUDIES IN INTACT ATRIUM:

The purinergic control of heart function has been shown by studies in intact organ (rev. by Pelleg et al., 1990, Burnstock, 1990). These studies provide less detailed cellular information than the ones on isolated cells, but may result in practical utilization of the results. Accordingly, studies of purinergic transmission in intact heart have already led to clinical use of adenosine in some types of cardiac arrhythmia (Greco et al., 1982).

As reviewed in the previous chapter, heart function can be regulated by neural and non-neural (endocrine, autocrine, cell to cell) purinergic mechanisms based on the source from which extracellular ATP is mobilized. Because of the strategic disposition of conduction nodes and nerves, atrial myocytes have different functions compared to ventricular myocytes. Atrial myocytes also differ from ventricular cells in their smaller size, their ability to secrete hormones, and the presence of different membrane receptors (rev. by Sheperd, 1992).

Studies of purinergic extracellular effects in intact atrium are complicated by the fact that ATP is the principal source of adenosine, and yet the two mediators have different receptors (Burnstock et al. 1990). Past evidence has indicated that in the whole heart most regulatory activity following an increase in extracellular ATP is mediated by adenosine (rev. Pelleg et al. 1990). In addition, purinergic stimulation of
contractile activity in intact atrium is mediated by atrial, nodal and ventricular purinergic receptors. An overall picture of cardiac purinergic receptors and purinergic effects on systemic cardiac functions is shown in tables 1 and 2. The tables show that extracellular purinergic receptors are present in all the cell types present in the heart. For potential clinical use, it is important to define which of these receptors participate in regulatory mechanisms relevant for a specific cardiac physiological or pathological activity. Since extracellular mediators may act together, it is also relevant to know what is the result of a concerted stimulation on cardiac function of purinergic and other extracellular agonists. The presence in intact organs of multiple receptors and cell types, makes pharmacological studies at this level difficult to interpret.

B. PREVIOUS WORK ON PURINERGIC CONTROL IN INTACT HEART:

Previous studies have shown the effect of purinergic mediators on intact heart functions at different levels: on coronary flow, on cellular metabolism and on electrical activity (rev. by Pelleg et al., 1990).

Effects on coronary flow and metabolism
During increased myocardial work and in any condition of high oxygen demand or in ischemia, ATP and adenosine are increased in coronary efflux (rev. by T. Forrester, 1990). This increase induces an increment in the myocardial blood flow (hyperemia). Ischemia/reperfusion induced coronary hyperemia is now believed to be mostly attributable to the
presence of adenosine (rev. by Engler and Gruber, 1992). It has been shown that adenosine decreases the ischemic damage, myocardial stunning, and infarct size (rev. by Hori and Kitakaze, 1991). Adenosine decreases several injury determining factors. Besides increasing central and peripheral coronary flux, adenosine limits potential damaging activities of leukocytes and platelets, Ca2+ overload, and catecholamines effects (by antagonism of the neural release and direct antagonism of catecholamine effects). Adenosine also shift the metabolic substrate of myocytes from fatty acids to carbohydrates (important in low oxygen). These protective activities occur at very low concentrations, while the electrophysiological effects take place at higher doses (Pelleg et al., 1990). The same antiischemic activities of adenosine have now been demonstrated in intestinal ischemia (Kaminski and Proctor, 1992). Adenosine acts through P1 receptors (Burnstock and Brown 1981) that are linked to adenylate cyclase in antagonistic (A1) or agonistic (A2) manner (Daly 1985). Both A1 and A2 subtypes are antagonized by methyl-xanthines. A2 receptors are located in coronary endothelia and mediate relaxation. Smooth muscle cells in this vessels also have P2Y or P2U ATP receptors which mediate relaxing activity. ATP, unlike adenosine, does not have a clear effect in ischemic damage protection. The reason of this difference could be related to stimulation of activities at levels other than coronary flow.

Effects on electrical activity
In intact heart, adenosine slows the sinus pacemaker spontaneous activity and the atrio-ventricular conduction, by inhibiting electrical propagation between region of the sinoatrial (SA) and atrioventricular (AV) nodes (rev. by Pelleg et al., 1990). In atrial myocytes and nodal cells, adenosine increases a potassium conductance of the cell membrane (Drury and Szent-Gyorgyi 1929, Hartzell 1979, Belardinelli and Isemberg 1983, West and Belardinelli 1985, Kurachi et al 1986, Takikawa et al. 1990). In atrial myocytes, adenosine causes a hyperpolarization, and a dose-dependent decrease in the action potential duration (Pelleg 1985). A1 receptors mediate adenosine activities in atrial and ventricular myocardium and in SA and AV nodes. These receptors are also located at presynaptic terminations, where adenosine decreases release of transmitters (Engler and Gruber, 1992).

ATP shares some of these activities when added in vivo, particularly control of the AV node conduction (rev. by Pelleg et al., 1990). Intravenous or intracoronary infusion of adenosine or ATP decreases canine heart rate after beta block and vagotomy (Pelleg et al. 1985). Most of this ATP effect has been attributed to hydrolysis of ATP to adenosine (Favale et al. 1985). Adenosine and ATP increase vagal tone and antagonize beta-adrenergic activity (Pelleg et al 1985). Adenosine reduces isoproterenol-induced arrhythmias (Rosen et al. 1983). Adenosine and ATP also block Purkinje fiber conduction if the fibers are previously stimulated by adrenergic agonists Lerman et al. 1987. Ventricular automaticity is also reduced. This effect reduces the rate of
ventricular escape and is mediated by adenosine receptors (Pelleg et al. 1986). Adenosine is particularly effective at low membrane potentials (Rosen et al. 1983). Adenosine reduces atrial action potentials, but has no effect on action potentials of isolated ventricular myocytes (Isenberg and Belardinelli, 1984). In contrast, ATP increases ventricular action potentials (Christie and Sheu, 1990).

These observations indicate that adenosine activity prevails on nodal cells and atrial myocytes. In these cells adenosine antagonizes adrenergic effects and increases vagal tone, while ATP effects are similar to those of adenosine or mediated through hydrolysis to adenosine. ATP may have some specific activity in ventricular myocytes where it increases the action potentials duration.

Because of the actions on cardiac excitability, adenosine and ATP are now commonly used clinically for terminating reentrant supraventricular tachycardia, which involves the atrioventricular node (Greco et al., 1982, Di Marco et al. 1983, Belhassen et al. 1983). It is assumed that the effects of ATP are accounted for by adenosine formation.

In later work, including the data reported here, evidence was produced that ATP effect on nodal and conduction cells and atrial myocytes is partially different from adenosine. Takikawa et al. 1990, showed that ATP induces SA nodal tachycardia when the effect of adenosine was antagonized. This effect might be mediated by diacylglycerol and prostaglandin release. The P2 receptor involved in this
response acts through a pertussis toxin insensitive phospholipase C pathway. These studies were conducted by recording the cardiac electrical activity in intact heart perfused on a Langendorff apparatus. Legssyer et al., 1988 and Scamps et al. 1990, have shown that selective P2 receptor activation in ventricular and atrial myocytes generates increased inotropy. Under both conditions adenosine receptors were antagonized by xanthines. Those two reports (still unpublished at the time of this study) indicate that ATP may have an inotropic effect on atrial and ventricular myocytes, under conditions where adenosine receptors are inhibited by antagonists.

The data presented in this study were obtained by measuring isometric tension in isolated rat atrium. They suggest that in rat atria the addition of ATP and adenosine decreases inotropism. This effect varies, depending on the previous addition of acetylcholine or norepinephrine, and is mostly mediated by adenosine. However ATP, unlike adenosine may moderately increase atrial contractile force if added after norepinephrine. With respect to the data of Scamps et al., this observation is novel because the potentiating effect of ATP is possible in presence of adenosine, produced by ATP hydrolysis, which antagonizes atrial contractile force.

Data are also shown on extracellular ATP regulated calcium transient in isolated atrial cells. Previous studies have shown that ATP-induced increase of calcium in ventricular myocytes is partially mediated by ATP activation of a non-selective cation conductance (De Young and
Scarpa, 1987, Christie et al., 1991, Zheng et al., 1992). A similar current has been reported in bullfrog atrial myocytes (Friel and Bean, 1987). The studies reported in this chapter show that ATP receptors can induce calcium transients in atrial myocytes. Calcium transients are induced by ATP but not adenosine and are potentiated by norepinephrine. The ATP effects on calcium intracellular homeostasis in atrial myocytes are similar to those in ventricular myocytes (De Young and Scarpa, 1987).
RESULTS

TENSION STUDIES:

Methods:

Male Sprague-Downley rats 300-400 g were sacrificed, the heart was removed and placed in Krebs Henseleit pH 7.4. All tissues, with the exception of auricles, were dissected and discarded. Threads were tied at the tip of each auricle and the preparation was mounted on an isometric force transduction device, recording as shown in Figure 1A. As shown in the figure, the atrium chamber (4.5 ml volume), was filled with solution, and the solution was changed through aspiration. O₂ was bubbled continuously in the solution. Interruption of O₂ resulted in more irregular beating. Prolongation of the anoxia would result in more pronounced irregularity, which worsened upon reoxygenation indicating an irreversible atrium damage. In condition of good oxygenation the atrium was beating regularly for 3-4 hrs.

The figure shows also that the bath was temperature controlled at 30⁰, since the temperature effect on the atrial beat was significant. Figure 1C shows a decrease in beating force and an increase in frequency by changing the temperature between 29⁰ and 36⁰. The force transduction device was attached to an amplifier and a strip-chart recorder.

Finally, figure 1B shows the effect of acetylcholine (Ach), or norepinephrine (NE). Since the atrium was spontaneously beating,
among different preparations, there was a large variability in rate and strength of contraction. This was taken into account in analyzing the trace by standardizing to NE or Ach effects. The presence of stripped neural terminations did not seem to influence the responses since addition of atropine 2 uM or propanolol 1 uM, had no effect on the initial tracing (not shown).

**Results:**

Figure 2 shows that the addition of ATP (100 uM) or adenosine (100 uM) to unstimulated rat atrium generated a large decrease in inotropism and frequency of spontaneous heart beat (chronotropism). This decrease progressively resulted in a complete block if adenosine was added after acetylcholine (0.9 uM). The block was reversed by high doses of norepinephrine (not shown).

These results are at variance with those reported by Takikawa et al. 1990 using the perfused intact rabbit heart. In those studies electrocardiographic activity was recorded, and in 50% of preparations ATP but not adenosine transiently increased the sinus rhythm. The ATP effect in those experiments was followed by a predominant adenosine-like response, similar to the one observed by us.

When 1 uM NE was added to the atrium, a large increase in tension and frequency of contraction was observed. If adenosine or ATP were added after NE, as shown in Figure 3, there was still a decrease in pacing and a less marked decrease in inotropism. However in some
atrium preparations we saw a slowing of the pacing and a transient small increase in inotropism upon addition of ATP but not adenosine (3 out of 8 with ATP, as compared to 0 out of 8 with adenosine). This increase was small, about 10%, and it is not observed with adenosine (Fig. 4). This increase was not seen after addition of the beta antagonist propanolol 1µM (or atropine 2µM) in all five preparations tested. These drugs by themselves did not influence the tension or the pacing-frequency. Hence, in the preparations showing potentiation of ATP and NE, the effect could be mediated through beta receptors.

We tried to use selective antagonists of adenosine, with the hypothesis that adenosine was masking a separate ATP effect in this preparation. We used 1,3-dialkyl-8-(p-sulphophenyl)xanthine, derivative of theophylline, antagonist of A₁ and A₂ (Daly et al., 1985) at the dose of 10 µM, and 8-cyclopentyl-1,3 dipropyl compounds, more specific for A₁ (Haleen, Steffen and Hamilton, 1987). Both compounds induced a time dependent toxicity in this preparation (at variance from perfused hearts) judged by the fact that the atrium became unresponsive to any stimulus, including acetylcholine and norepinephrine. The toxicity might be accounted for by the formation of free radicals by these compounds in the presence of oxygen.

Conclusions:

These data yield more qualitative than quantitative results because of the high variability between the preparations used and sometime within
the same preparation. Still, they indicate that in isolated atria the composite response of conduction and myocardial cells is by large attributable to adenosine effects, or alternatively, to ATP effects which are similar to adenosine (as previously reported rev. in Pelleg et al., 1990). These effects are dependent on the presence of Ach or NE. In particular, adenosine and ATP potentiate Ach and antagonize NE effects on inotropism and automaticity of the atrium. Therefore ATP and adenosine in the intact atrium antagonize the adrenergic system and potentiate the vagal tone.

At variance from Hori et al. which studied the electrical activity of rabbit hearts, we failed to observe a transitory ATP induced increase in pacing (looking at atrial contraction). However, in presence of NE, ATP but not adenosine increases inotropism in 38% (3/8) of the preparations. The NE effect is likely mediated through the activation of beta receptors.

Scamps et al., 1990 have shown that in presence of beta-1 stimulation, ATP increases inotropism in auricles. In the experimental conditions used in that work the effect was unmasked by pre-treatment with pertussis toxin, which eliminates some of the effects of adenosine, such as the adenosine dependent activation of potassium current in atrial myocytes.

It is known that ATP increases contractile force in ventricular cells, (Legssyer et al., 1988), and ATP generates calcium transients in ventricular myocytes (De young and Scarpa, 1987). The magnitude of the Ca\(^{2+}\) transients is increased by NE. The increase in calcium could be
the intracellular signal of the increased inotropism in ventricular and atrial myocytes. Fura-2 loaded isolated atrial myocytes were studied to establish if calcium transients could be induced by ATP in these cells.

**ISOLATED ATRIAL MYOCYTES:**

**Methods:**

Cells were separated as described in De Young, Giannattasio and Scarpa, 1989, through a procedure designed to isolated functional myocytes. Nembutal anesthetized rats (150-200 gr) were sacrificed, the heart was removed and mounted on a modified Langendorff apparatus. The heart was perfused at 10 ml/min using Joklik modified Eagle's medium, supplemented with CaCl₂ (1.25 mM) and Hepes (10 mM), pH 7.2 for 5 min, followed by 5 min in a similar medium containing no added calcium. Type I collagenase (100 U/ml Cooper Biomedical) with 0.1% BSA was added after 20 min to initiate the digestion and continued for 45 min. To avoid calcium toxicity calcium was added back at successive intervals of 5 min to yield final concentrations of 0.25 mM, 0.50 mM, 1 mM, 1.25 mM. The atrium was then dissected and minced in a Petri dish containing Ca²⁺ Joklik and fresh collagenase, and incubated for 5 min under O₂ in a water bath at 37°C. Cell viability was determined as described in the above mentioned reference (shape, % cells beating, trypan-blue loading, response to depolarization induced by extracellular potassium, nucleotide content). Viable preparations of cells were loaded
with Fura2-am for fluorimetric determination of intracellular calcium. Atrial
cells appeared smaller than ventricular cells obtained using the same
protocol.

Fura2 fluorescence measurements were made as described by De
Young and Scarpa, 1987. Cells were loaded with 2 uM Fura2-am for 40
min at 37°C in Ca2+-joklik medium and then washed with the same
medium. An aliquot, usually 10^4 cells, was resuspended in 2 ml Geigy
medium, and used for fluorescence measurements. A major limitation in
these experiments was the number of cells, since only 2-3 measurements
were possible from the cell yield of a single heart. Fluorescence was
determined in cuvette, at 37°C, with constant stirring in a custom-built
fluorimeter designed by the University of Pennsylvania Biomedical
Instrumentation Group. Excitation (339 nm) and emission (490 or 510
nm) wavelengths were established by interference filters (Omega Optical,
Brattleboro, Vt). Traces were recorded on a strip-chart recorder. From
the fluorescence signal, calcium was calculated as described by
Grynkieciewicz et al., 1985, from the equation F-F_{min}/F_{max}-F \times 224 \text{ nM} =
[Ca2^+]. The F_{max} was determined upon addition of digitonin, and the
F_{min} by addition of saturating amount of EGTA. In most cases Ca^{2+}
transients were measured as the ratio between the signal obtained with
ATP and that obtained by addition of 30 mM KCl to the same cell
suspension. The expression of the data as a percent of the KCl response
allows a comparison between different samples of cells. The exact
quantification of the signal according to the above equation, is often
mistaken because of compartmentation dependent errors in the determination of \( F_{\text{max}} \) and \( F_{\text{min}} \).

**Results:**

A typical calcium transient is shown in Figure 5A. Addition of 25 \( \mu \)M ATP generates a transient increase and the addition of 35 mM KCl a larger stable increase in intracellular Ca\(^{2+} \). The effect of ATP is increased if NE 2 \( \mu \)M is previously added (Figure 5B) (n 3).

Adenosine (25-100) did not generate a transient (n 3), (not shown).

In conclusion ATP induces in atrial myocytes a Ca\(^{2+} \) transient which is potentiated by norepinephrine. This effect is specific for ATP as compared to adenosine and is similar to the one seen in ventricular myocytes. This effect could be involved in the ATP induced increase in inotropism observed in the intact atrium.
Figure 1:

1.A: Atrium isometric force measuring device. Atria were beating in absence of electrical stimulation 1.B: Effect of 1 µM norepinephrine (NE) and acetylcholine (ACH) on atrial tension. The addition of drugs is indicated. In this and the following figures tension is expressed in arbitrary units. Time is shown in sec. 1.C: Effect of temperature on tension. Temperature of the chamber wall was regulated through a water bath.
A. Tension measuring device

B. Effect of NE and ACH on tension

C. Temperature effect on force

Fig. 1
Figure 2:
Negative inotropic and chronotropic effect on atrial tension induced by 100 uM ATP or 100 uM adenosine. The additions are indicated in the figure. Traces are from right to left, paper speed was 15 cm/min.
Figure 3:
Effect of 100 uM ATP or 100 uM adenosine after the atrium was pretreated with 1 uM NE 1 min before (trace shown is after NE addition).
Fig. 3

100uM ATP

A

1uM NE

B

100uM Ado

1uM NE

Time in seconds

Tension (arbitrary units)
Figure 4:
Positive inotropic and negative chronotropic effect on the atrium upon addition of 300 uM ATP after 3.2 uM NE, as indicated in the figure.
Fig. 4

Tension (arbitrary units)

Time in seconds

300 μM ATP

3.2 μM NE
Figure 5:
Time dependent changes of the fluorescence emission at 490 nm of two suspensions of isolated atrial cells (5A and 5B) loaded with the calcium-sensitive fluorescent indicator Fura2-am. Excitation was 339 nm. Cells were tested in figure 1A with 25 uM ATP and 30 mM KCl. In figure 1B with 2 uM NE, 25 uM ATP and 30 mM KCl. The response to KCl is used to compare the different cell suspensions. All other conditions are described in the text.
Fig. 5

KCl
ATP 25 uM
NE 2uM

KCl
ATP 25uM

(arbitrary units)

light emission at 490 nm

1 min
### TABLE 1

**Purinergic Receptors in Different Tissues of the Heart:**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Adenosine Receptors</th>
<th>ATP Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrial Myocytes</td>
<td>A1</td>
<td>P2</td>
</tr>
<tr>
<td>Ventricular Myocytes</td>
<td>A1 (?)</td>
<td>P2</td>
</tr>
<tr>
<td>Sinus Node cells</td>
<td>A1</td>
<td>P2y</td>
</tr>
<tr>
<td>AV-Node cells</td>
<td>A1</td>
<td>ND</td>
</tr>
<tr>
<td>His Bundle</td>
<td>A1</td>
<td>ND</td>
</tr>
<tr>
<td>Coronary (Endothelium)</td>
<td>ND</td>
<td>P2</td>
</tr>
<tr>
<td>Coronary (Smooth muscle)</td>
<td>A2</td>
<td>P2y</td>
</tr>
</tbody>
</table>

### TABLE 2

**PURINERGIC ACTIVITIES ON PRINCIPAL CARDIAC FUNCTIONS:**

<table>
<thead>
<tr>
<th>FUNCTION</th>
<th>ADENOSINE EFFECT</th>
<th>ATP EFFECT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary Dilation</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SA chronotropic effect</td>
<td>-</td>
<td>+ then Ado-</td>
</tr>
<tr>
<td>AV-node velocity</td>
<td>-</td>
<td>- Ado</td>
</tr>
<tr>
<td>Action potential (Atrium)</td>
<td>-</td>
<td>? +</td>
</tr>
<tr>
<td>Action potential (Ventric) no</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inotropism (Atrium)</td>
<td>-</td>
<td>+ then Ado-</td>
</tr>
<tr>
<td>Inotropism (Ventric) no</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Prostanglandin release</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Adrenergic antagonism</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Reduced ischemic damage</td>
<td>+</td>
<td>?</td>
</tr>
</tbody>
</table>

+/-: positive or negative effect on the function; - Ado: ATP effect is not proven and effect of adenosine derived from ATP prevails; + than Ado -: ATP positive effect is followed by adenosine negative effect, which prevails; no: no effect.

CHAPTER 3

FUNCTIONAL EXPRESSION AND PROGRESS TOWARD EXPRESSION-CLONING OF CARDIAC ATP RECEPTORS
ABSTRACT

This chapter shows the results of functional expression in Xenopus Laevis oocytes and the progress toward cloning of one of the cardiac ATP receptors inducing activation of cationic currents and calcium transients in ventricular myocytes. This work and the one reported in the next chapter characterize two cardiac ATP receptor types. The existence of two different ATP receptors has been proposed on the basis of previous experiments (De Young and Scarpa, 1991). These experiments have shown two functional mechanisms, possibly mediated by separate receptors: one responsible for activation of ATP induced cationic currents and the other for ATP induced Na/Pi transport (see chapter 1). In this study both receptors were expressed from cardiac mRNA size-separated fractions. Preliminary experiments show similar functional expression from a cDNA library made from total cardiac mRNA. In this chapter is shown the expression of the receptor responsible for the ATP induced cation conductance, while in the next chapter is shown the labelling of two ATP binding proteins, tentatively identified as the two cardiac ATP receptor types mentioned above. Data on the expression of the ATP induced NaPi transport will be presented in a separate manuscript (Powers, Giannattasio and Scarpa, in preparation).
INTRODUCTION

Extracellular ATP elicits a variety of receptor-mediated responses in many different cell types, the nature of which is determined by the subtype of P$_2$-purinergic receptor activated and the second messenger possible system involved in this activation (Burnstock, 1990, Dubyak, 1991). In rat myocardial cells, evidence supports the presence of an ATP receptor that act as a ligand-operated non-selective cation channel (De Young and Scarpa, 1987, Friel and Bean, 1987, De Young and Scarpa, 1989, Zheng et al., 1992, Christie et al., 1991, 1992). In quiescent isolated myocytes, occupancy by MgATP of this receptor depolarizes the cells, thereby activating L-type calcium channels and inducing a transient increase in cytosolic free calcium. Calcium influx directly through the cation channel is also part of the ATP induced calcium transient (De Young and Scarpa, 1989). The receptor is not activated by the anionic form of ATP and has an unique ligand specificity (2-methylthio-ATP (2-me-S-ATP) > ATP > ATP$_\gamma$S) with little or no response to other nucleotides including the usual activators of P$_{2x}$ channels or UTP. Extracellular ATP has been shown to activate an inwardly directed Na/Pi cotransport in rat myocytes, and because of the increase in cytosolic sodium, cell calcium increases via Na/Ca exchange (De Young and Scarpa, 1991). However this effect, unlike the rapid calcium response, occurs over min rather than sec after ATP addition, and with a different ligand specificity (ATP$_\gamma$S >
ATP >> 2-me-S-ATP). These differences suggest the presence of at least two subtypes of cardiac ATP receptors.

A comprehensive picture of Ca\(^{2+}\) modulation mediated by the two cardiac ATP receptors and transduction system described above is diagrammatically illustrated in Figure 1.

A detailed understanding of ATP receptor types, structure and function is still hampered by the absence of receptor isolation. The absence of specific receptor ligands and an abundance within the plasma membrane of several ATP-binding proteins limit protein purification approaches. As an alternative method, we utilized functional expression of myocardial ATP receptors in Xenopus Laevis oocytes injected with cardiac mRNA. By using a photo-luminescence assay for detecting cytosolic calcium transients, we identified a functional response to ATP which induces cationic currents in ventricular myocytes. This method may allow indirect characterization of the transduction mechanisms activated by ATP receptors and, potentially, the isolation of the cDNA coding for the ATP receptor.
METHODS AND RESULTS

A. FUNCTIONAL EXPRESSION

Preparation of mRNA:

mRNA was prepared from either intact rat heart or isolated myocytes. Intact heart was more frequently the method of choice, since it produces higher amount of RNA and less mRNA degradation. Hearts were rapidly isolated from rats, frozen in liquid nitrogen and stored at -80°C. A preparation usually consisted in a pool of 5-10 rats (5-8 gr of cardiac tissue). RNA isolation was carried out after lysis of myocytes in an environment resulting in fast denaturation of ribonucleases with precaution against contamination from solutions and environment (Sambrook et al., 1989). Three procedures of mRNA isolation were tested: the "Fast Track" isolation kit (Invitrogen, California); the centrifugation on cesium gradient (Sambrook et al., 1989); the thiocyanate-phenol-chloroform (acid phenol) extraction (Chomczynski and Sacchi, 1987). The Invitrogen kit is a batch preparation, which means that for mRNA separation it utilizes oligo (dT) resin in suspension with the total tissue extract. This preparation, unlike the other two, gives directly mRNA from the tissue extract, but results in higher contamination by proteins, DNA and other RNA species. The cesium gradient preparation produces total RNA, of good quality but more easily degraded than that derived from the following method. The thiocyanate-acid phenol method gave the best quality mRNA as controlled by RNA gels. This method of
mRNA extraction utilizes strong protein denaturing conditions at all the steps of the purification. Using this latter preparation, 8-10 gr of cardiac tissue yield approximately 5-10 mg of total RNA. With either of the last two methods Poly(A⁺) mRNA could be obtained by purification on oligo (dT) cellulose column in a RNase free environment. The protocol used is as described by Sambrook et al., 1989, using the optimized ratio of oligo (dT) in the column to total RNA amount (a very important condition). mRNA yield was, after two consecutive purification steps on the column, between 300-600 ug of mRNA, from 5-10 mg of total RNA. mRNA was then stored as ethanol precipitate. The conditions of mRNA precipitation were described by Sambrook et al., 1989.

The mRNA, prepared as described above, was used for in vitro translation and for oocytes injection. The "first strand" synthesis in library making (see below), requires further purification steps to separate from contaminants derived from the cells or the solutions used in the previous steps of isolation. As judged from the agarose gels of "first strand" cDNA, the reverse transcription reaction used in library making was improved if the mRNA was sedimented through 0.22 micron filters which were loaded with small amounts of preactivated oligo (dT) resin. This "minicolumn" step was followed by extraction of the eluent with phenol and ethanol precipitation.

*Fractionation of mRNA:*
Poly(A)$^+$ RNA (about 200 ug) was fractionated by centrifugation on sucrose gradients in presence of methyl-mercuric hydroxide, which denatures and linearizes mRNA. Under these conditions mRNA molecules separate on the gradient according to their number of bases. The gradient centrifugation was made as described in Sambrook et al., 1989. Fractionated mRNA was recovered in aliquots (usually 30 of 0.4 ml each) and each mRNA fraction was ethanol-precipitated. The precipitated mRNA fractions were resuspended in sterile water (RNase free), measured by absorbance at 260/280 nm and, after proper dilution, were injected into oocytes. A small fraction from each band was evaluated for size in a denaturing gel (agarose gel containing formaldehyde or methylmercuric). In few experiments the correctness of the gradient was checked by refractometry. A typical example of the gradient is shown in Figure 4A.

**mRNA quality control:**

In some experiments mRNA quality was checked. As a control of degradation, mRNA was transferred to nitro-cellulose membrane and analysed by a standard protocol as described in the Genescreen Plus manual, Dupont Wilmington, DE, and alternatively by a similar protocol given to us by Dr. Mattera, CWRU Cleveland, OH. Radioactive human actin probes were prepared by random primer labelling. mRNA degradation was analyzed by detecting hybrids formation between the probes and the actin mRNA of our preparation.
The ability of the mRNA to induce protein translation in vitro was also investigated. The rationale for this control is that if the in vitro preparation is able to translate the extracted mRNA, oocytes, which are about 100 times more efficient, might translate it. Translation in vitro was done by using reticulocyte lysates kits (Amersham Corporation, Illinois or Boehringer Mannheim, Indiana), according to their standard protocols. Proteins produced by the reaction were labeled by adding $^{35}$S-methionine in the reaction mixture. Label incorporation was measured by scintillation counter after filtering. mRNA specific translation was measured as compared to the amount of incorporation induced by sterile water (no template) or by a purified viral mRNA, which is a very efficient template.

*Xenopus Laevis* oocytes as a translation device:

Recent studies (rev. by Dascal et al., 1986, Dascal, 1987, Snutch, 1988) reveal that *Xenopus* oocytes provide a powerful system for purifying a cDNA encoding a receptor and analyzing receptor structure and function. The main reasons are the following: 1) oocytes have capacity (and reserve) for translation of injected exogenous mRNA. 2) the product of translation is in most cases fully functional. Therefore even a message present in a small number of copies, such as a membrane receptor, can be synthesized and functionally expressed. 3) the oocyte has well characterized membrane properties and intracellular messenger activities. 4) oocytes are stable cells that survive invasive manipulation.
Preparation of oocytes and injection of mRNA:

Fully grown oocytes (stage 6 of Dumont) from Xenopus laevis were isolated from the frog ovary by dissection. Oocytes are surrounded by several layers of follicular cells. Since mechanical removal of follicular cells reduces viability and increases membrane permeabilization, defolliculation was carried out by an enzymatic treatment (2 mg/ml of collagenase, type V or IA Sigma were used without detecting difference) followed by mechanical dissociation (Dascal 1987). Collagenase digestion was carried out in Ca$^{2+}$-free modified Barth solution (MBS) (Dascal, 1987). Cells were then transferred in regular calcium MBS and separated manually. After 5-10 hours, viable-looking cells were separated and transferred to clean MBS solution. The cells were injected with mRNA solution (up to 50 ng of poly(A)$^+$ mRNA) in volumes ranging between 20 and 50 nl. In control experiments it was found that amounts of mRNA greater than 100 ng impair the transcription ability of healthy oocytes. This limit was determined by injecting oocytes with substance-K receptor mRNA (Dr. S. Nakanishi) and detecting the signal generated by the expressed receptor. When is expressed in oocytes, this receptor generates intracellular calcium release upon addition of substance-K (see below). These experiments were done by injecting constant amount of single clone mRNA plus a variable amount of cardiac extract mRNA.

Oocytes can be maintained viable for 6-7 days after injection. In control experiments using injection of single clone of substance-K
receptor or of mRNA fractions which gave positive response to ATP, it was found that the optimal time for the response occurred 2-3 days after the injection. Injection of the mRNA was done under microscopic observation using a compressed air microinjector (WPI, Florida). It was found that injection in the animal pole of the oocytes preserved cell integrity and improves transcription.

ATP (and adenosine) endogenous receptors are present in oocytes, however they are lost upon defolliculation treatments (Dascal, 1987), like the one described above. In our condition it was verified that uninjected or water injected oocytes were not responding to ATP.

**Functional Assays:**

Our expression procedure was designed to increase the possibility to express and isolate the message coding for the ATP receptors, which mediate cation influx in ventricular myocytes and induces in these cells a transient cytosolic calcium increase (De Young and Scarpa, 1989). This receptor might be a ligand-operated cation channel (Christie et al., 1992, Zheng et al., 1992). The ATP dependent cation influx may activate L-type calcium channels through depolarization and Ca$^{2+}$ dependent release of calcium from the sarcoplasmic reticulum (De Young and Scarpa, 1989, Christie et al., 1992, Zheng et al., 1992). Several methods were used for expression of the ATP dependent calcium tansients. The one which yield more promising results was the detection of calcium-induced luminescence of aequorin. Aequorin is a jellyfish protein which emits
photons upon calcium binding and it is a sensitive calcium indicator (provided by Dr. Blinks, Rochester, Mn). Cells were loaded with aequorin and tested for extracellular ATP response in high extracellular calcium (see below). In oocytes injected with total cardiac mRNA, this assay might detect an intracellular calcium increase derived from the ATP induced direct influx through the cation channel, or from mechanism of calcium release from intracellular stores. As shown below, fractionation of the mRNA message resulted in a larger signal from a size selected mRNA which is smaller than cardiac channels or cardiac ryanodine receptor message. This result suggest that the aequorin assay is sensitive enough to detect direct ATP induced influx of calcium after the relevant message is relatively concentrated by fractionation. An alternative explanation is that oocyte endogenous calcium channels participate to the ATP induced calcium transient.

Other assays tested are: 1) direct recording of ATP induced current by electrophysiology; 2) recording of oocytes endogenous Cl currents stimulated by intracellular calcium increase (Dascal, 1987); 3) ATP induced influx of labelled cations. The first method directly detect the ATP induced cation conductance. The second method looks at an amplified signal down stream from the activation. This method might require a large change in intracellular calcium concentration as the one derived from intracellular release or voltage dependent calcium channels activation (Dascal et al., 1987). This method has been successfully used for expression of ATP receptors linked to intracellular release of calcium
(Nuttle et al., in Press). The first two methods did not give any ATP dependent signal in oocytes injected with total or fractionated mRNA heart message. In the same condition, injected message coding for substance-K receptor, which induces release of intracellular calcium, was efficient in producing Ca\(^{2+}\) activated Cl\(^-\) currents (not shown). These experiments were conducted by voltage clamping the oocytes between -50 and -60 mV with two intracellular electrodes attached to a Axoclamp-2A clamp instrument (Axon Instr. Inc., California). ATP was added in the extracellular solution, either in regular MBS or in solution containing high calcium (2-3 mM).

Radiolabelled ions transport measurements were used in the expression of a separate cardiac receptor, inducing ATP and ATP\(_7\)S activated Na-Pi co-transport (Powers, Giannattasio and Scarpa, manuscript in preparation).

It is possible that in oocytes the newly expressed ATP receptor has a different coupling than in cardiac myocytes. It is also possible that more than one factor or subunit are required in the functional expression. Some of these potential hindrances may be prevented by the strategy of clone isolation, but definitive answers to these hypotheses can be obtained only upon success in receptor isolation.

Results of Photoluminescence Assays:

Oocytes were injected with the calcium indicator aequorin (stock solution of 1.5 mg/ml in 10 uM EGTA). Since Rnase activity was found in
the aequorin solution, the indicator was injected separately from the mRNA a few hours before testing to minimize mRNA degradation.

In order to have maximal influx of Ca\(^{2+}\) through the cardiac cation conductance and avoid ATP mediated activation of Na/Pi transport, assay solutions contained 15 mM Ca\(_2\)Cl, 110 mM Sucrose, 1 mM Mg\(_2\)Cl and 10 mM Hepes buffered to pH 7.5. High concentration of MgATP (1-2 mM) is required for ATP agonism in this solution, since the calcium-bound form of ATP is inactive (De Young Ph.D. Thesis, 1990). Concentrations of the magnesium-bound and free ATP forms were calculated from a computer program designed at the Brain Research Laboratory, University of Texas, San Antonio. Decrease of Ca\(^{2+}\) to less than 15 mM in absence of other ions, makes the cells very unstable. MBS solution with 2-3 mM Ca\(^{2+}\) or MBS solutions in which NaCl was substituted with TRIS-Cl or NMG-Cl were also tested. In both conditions the signal from total or fractionated injected mRNA was not easily distinguishable from control.

Single oocytes injected with mRNA and/or aequorin were tested in cuvette in a specially designed metal basket. The signal was detected by using a photomultiplier and amplifier systems (Pennsylvania Bioinstrumentation, PA), linked to a strip-chart recorder. Cuvette volume was 1.5 ml, under constant stirring. The test was started by addition of ATP after few minutes ionomycin was added. This calcium ionophore induces, under these conditions, Ca\(^{2+}\) influx and therefore a large response in aequorin response is observed. If no or small response was observed,
this was an indication of either lack of sufficient aequorin injection or aequorin irreversible previous exposure to high cytosolic calcium.

Figures 2A and 2B schematically illustrate the mRNA preparation and the detection system. As shown in the figure, the cuvette holding the oocytes was contained in a light-tight box, which was opened briefly for all the additions. For some experiments a flow system for ATP addition was designed and used, to avoid opening the light tight box. This system yielded no appreciable difference.

Figure 3 shows an example of detection of positive expression using this system. Injection of total rat cardiac mRNA into Xenopus oocytes results in the expression of cardiac ATP receptor as demonstrated by the increase in aequorin luminescence after the addition of extracellular ATP (Fig. 3C). Cells injected with aequorin alone or aequorin and substance K, did not respond to extracellular ATP (Fig.3A), but were still strongly positive to the addition of the calcium ionophore ionomycin. Cells that responded to ATP did not respond to similar concentrations of GTP (Fig.3B), UTP, or ADP (not shown), consistent with the specificity of the heart receptor to these agonists. A few oocytes were damaged by the injection procedure and were discarded on the basis of an unstable baseline or by the lack of response to the calcium ionophore ionomycin.

Size fractionation of denatured rat heart mRNA by sucrose density gradient centrifugation resulted in the concentration of ATP
receptor expression within pool 2, 3 and 4 (shown in Figure 4B). Peak response within this group occurred with injection of pool 3 mRNA.

Since every pool constituted the collection of three consecutive fractions, we injected the fractions constituting the pool and again as expected we saw higher expression from the mRNA of the fractions constituting pool 3 (pool resulting in peak response) as compared to the fractions constituting pools 2 and 4.

From these experiments and methyl-mercuric agarose RNA gels of the corresponding fractions the range of ATP receptor activity was determined to be in the range of 1.5 - 3.0 kb. This determination was done from linear regression of the distances on an mRNA gel, as compared to known mRNA standards.

ATP receptor expression became more reliable and consistent using size-selected mRNA. In three successful expression experiments we made a quantitative comparison of the percent of positive cells in the luminescence assays between total heart mRNA and size fractionated rat heart mRNA injected oocytes as shown below. Cells which did not respond to ionomycin or with an unstable baseline were not considered. In the above mentioned experiments, of 24 cells injected with total mRNA, 57% responded to ATP. Of 26 cells injected with the positive fraction of mRNA, 75% were positive to ATP. Including all experiments in which cells were actively transcribing substance-K, the percent of cells injected with total heart or fraction mRNA having a positive response was about 15% and 30%, respectively. In some of these experiments the quality of mRNA
was only controlled through agarose gel. In batches of cells which were not transcribing the substance-K message, there were no cell responding to ATP upon cardiac mRNA injection. These batches of cells were considered non actively translating.

The results of size determination experiments were confirmed by several fractionation (n5) from different heart preparations, or by repeated injections at different time of positive mRNA fractions from the same preparation. Possible artifacts due to mRNA induced calcium leakage were excluded because mRNA concentration was adjusted so that the amount of mRNA injected with different fractions was identical. The relative reproducibility of the responses in positive fractions allowed a more significant pharmacological characterization. In these preparations negative response to UTP and GTP, and the positive response to ATP confirmed the specificity of the expression of an ATP receptor.

An alternative detection system potentially faster in screening, was also used. In this case, ATP-dependent intracellular calcium increase in mRNA injected oocytes containing aequorin, was measured simultaneously in dozens oocytes. In a typical experiment, 100 oocytes injected with mRNA fractions or controls, were kept in separate microwells and then placed in the dark over a manifold containing a negative film. First, a control was done by adding water to every wells and developing the first film. Successively ATP, was added to the wells and aequorin luminescence appeared in oocytes where the cytosolic Ca^{2+} concentration increased. After 10 min this second film was processed.
and the exposed spots were considered representative of positive expression. Responsive single cells appear as separate dark spot on the film. A third film was added and, 3 min after addition of ionomycin, was exposed, to ensure that all the cells were responsive. This system was successfully used for expression of ATP response from total and fractionated mRNA (data not shown). However, while all the fractions that were positive with the previous method were also positive with this method, with this method we observed false positive. Hence, although this method is probably more sensitive, because measures a signal integrated over several minutes, it may lack adequate specificity.

Specificity of ATP expression:

The expression of the ATP receptor from total rat heart mRNA was variable. The variability appears dependent on the mRNA preparation, and on the status of the oocytes and/or the frog from which the oocytes are derived. These potential problems were controlled in the following way: 1) mRNA preparations were assayed for degradation through agarose gel and in some cases through Northern or translation in vitro. 2) the capacity of transcription of different oocytes batches was verified by substance-K expression. The substance-K receptor is a G-protein linked receptor which determines intracellular calcium release. Injection of extremely low number of copies of this receptor mRNA (fimol) in actively transducing oocytes, generates an increase in aequorin luminescence after addition of substance-K (10 nM) (Fig.3B). Oocytes injected with
mRNA coding for substance-K receptors could be separated in active and inactive batches on the basis of more than 100 experiments (about 10 cells each). In active batches about 90% of intact cells were responsive to SK addition. In inactive batches (about 40%) no cells were responsive to substance-K. Between the cells of a responsive batch, there was large variability of the amplitude of the signal detected: about four times (or more since aequorin signal is not linear) difference between cells injected with equal amount of message. Hence, cell batches can be identified based upon substance-K receptor expression, as translating and not translating. The difference between the two groups is large enough to assess presence or absence of translation in batches of oocytes where ATP expression was attempted. No ATP response was found in water injected or substance-K injected oocytes in active or inactive batches (n 10 batches). Batches non actively translating (judged from substance-K expression) did not express ATP response in cells injected with cardiac mRNA. However many batches where there was substance-K receptor expression, did not express ATP receptors when injected with total mRNA. In actively translating batches of cells injected with the specific mRNAs, 90% of cells expressed substance-K receptor, and only about 15% expressed ATP response (30% of cells injected with fractionated mRNA). Part of the reason for the difference between the success of expression of ATP and substance-K could be accounted for by a variability in quality of mRNA for translation, even in presence of the controls mentioned above.
A posteriori, a better control of translation in oocytes could be the expression of another cardiac molecule, e.g. a channel, from the total cardiac mRNA. This expression could then be compared to the ATP response. Preliminary attempts of expressing voltage-dependent calcium channels, by injecting total cardiac mRNA and using the aequorin assay, have not been successful. Depolarization with KCl generates a larger calcium signal in cells injected with water and aequorin, than in cells injected with mRNA and aequorin. In addition, it was found by intracellular electrode recording that after the injection of aequorin (which is done 2 days after the first injection), the membrane potential of cells injected with mRNA was more depolarized (-20 mV, preliminary measurements). This could be the reason of the smaller KCl response in mRNA injected cells than in water injected. This result also indicates the presence of endogenous calcium channels. 3) The aspecific or RNA induced leakness was, to a certain degree, controlled by the addition of ionomycin as described above. mRNA injection determines a certain tendency to leakage, which is dependent on the oocyte preparation and on the amount of mRNA injected. Hence, a proper control is not an oocyte injected with water, but one injected with equal amount of mRNA derived from receptor expressing or not expressing fractions. Even this control may not be perfect, since some mRNA fractions induce more leakage (and poor cell viability) than others, possibly for reasons related to the message in itself.
B. PRELIMINARY EXPERIMENTS OF EXPRESSION CLONING:

_Cardiac cDNA library:_

In order to isolate the gene responsible for the ATP responses we made several cDNA libraries representative of the rat cardiac mRNA. Because the libraries were used to attempt the expression cloning of a receptor, they must meet some specific requirements. The method selected for the expression is transcription in vitro, as compared to the previously described techniques employing hybridization between mRNA and library cDNA (Lubbert et al., 1987). This method was also selected because the frequency of expression was probably not sufficient to allow the evaluation of a positive hybridization technique. For the in vitro transcription technique, it is necessary that the cDNA contains the intact message for the receptor molecule or its functional part. Also, since usually receptors are not highly represented in the genome, it is necessary to have a large representative library, with high multiplicity (number of original clones). In addition, the transcription in vitro system requires that the cDNA is inserted in a cloning box having a specific promoter activity, so that mRNA can be synthesized. Finally, a lambda derived library will provide high multiplicity and large inserts average size. By contrast, a plasmid library would be much easier to handle in term of the DNA preparations and of mRNA in vitro synthesis.

We used a cDNA "Zap synthesis" kit and a lambda-unizap vector system (Stratagene, Ca). This vector met several requirements. 1) The
cDNA synthesis is directional, determining the formation of a molecule that can be inserted in a specific orientation in the lambda. In this manner, we could make only the "sense" message by mRNA in vitro synthesis, obtaining 100% efficiency, as compared to 50% in a non-oriented library. 2) This vector had been successfully used in oocyte for the expression cloning of a serotonin ligand-operated channel and of other molecules. 3) The vector is available with a developed system of "packaging" (Stratagene, Ca), to synthesize the active bacteriophage. This system allows the synthesis of libraries of high titer of original clones (multiplicity). In fact our primary libraries multiplicities ranged between 1,000,000 and 2,000,000 (tested titering by bacterial infection) from the size selected cDNA. 4) The vector contains a plasmid bluescript, which contains the cloning site, and which can be excised in vivo (meaning not by enzyme cutting, but by infection with specific helper phage, in specific bacterial cultures). Hence, the plasmid can be obtained after the library is made using the phage.

cDNA synthesis:

The cDNA synthesis protocol described by Stratagene was modified to enhance efficiency. Libraries were made from total mRNA, since a method to efficiently size select the double stranded cDNA was developed. The mRNA purification step mentioned in a previous section in this chapter was necessary for every reverse transcriptase enzyme used. We found a wide difference among reverse transcriptases
commercially available. A combination of the BRL superscript Moloney virus (M-MuLV) and the Boehringer Avian Myeloid Leukemia virus enzyme (AMV) was selected. In the second strand synthesis, a combination of RnaseH, E. Coli Ligase, and Dna Polimerase I also from BRL, was used. The blunting was done with a T4 DNA polymerase from Amersham. Also after trying several methods of cDNA size selection, including Sephacryl size selection columns and Schleicher and Schuell glass beads extraction from agar-gel, we developed a modified method of cDNA intercept on DEAE membranes (Schleicher and Schuell Na45 membranes), which gave the most efficient cDNA size selection and purification for successive ligation. All these steps are diagrammatically illustrated in Figure 5. The conditions for first and second strand cDNA synthesis and the protocol for DEAE cellulose DNA intercept are shown in table I. All other conditions for synthesis were similar to the Stratagene protocol described in the ZAP-cDNA synthesis kit. In this manner we synthesized 3 different rat cardiac libraries (2 from fractionated mRNA and 1 from total mRNA and size selected cDNA). We also synthesized a library from PC12 cells mRNA for comparative experiments. Average multiplicity of these libraries was 1,000,000 to 2,000,000 and average insert size varied from 1.4 to 2.5 kb. The libraries were amplified to an average of $10^{10}-10^{12}$ clones and pooled for successive cloning steps.

Cloning steps:
One of the libraries (F1/F2) has so far been utilized in an attempt to obtain the single clone for the ATP receptor expressed in the assays. The first step was to obtain a valid but rapid lambda preparation for isolation of the lambda DNA and successive cutting of the cDNA for mRNA transcription. We utilized: 1) simple plate preparation (Maniatis Protocol) with additional steps of phenol/chloroform extraction; 2) commercially available kits like Quiagen columns (Quiagen) and Gene clean (Promega) preparation; 3) a large scale preparation from a mixture of plates and liquid cultures. The latter (given to us by Dr. Mattera, CWRU Cleveland) involves several steps, yields more purified material, but requires several days for each preparation. The commercially available kits were not adequate, since they are essentially designed for plasmid preparations. In the preliminary attempt we used the plate lambda preparation. Preparations were evaluated on agarose gels as previously described (Sambrook et al., 1989). In the same way the ability of the purified material to be substrate of the required restriction enzymes was also tested. cDNA from amplified pools was prepared as above described and mRNA was made in vitro with an mRNA "capping" kit (Stratagene, Ca). Controls were performed by adding traces of radioactive nucleotides in some reactions and evaluating newly transcribed mRNA on gel.

A preliminary attempt of clone isolation according to a strategy similar to the one reported in Sambrook, 1989 was made (Fig. 6). In following steps, progressively smaller pools of library clones were tested by lambda cDNA preparation, in vitro transcription of mRNA and mRNA
injection in the oocytes. In the experiments shown in the Figure 6, pools were tested usually about three times (10 cells for each pool, three times) and in every step the pool having more positive percent expression within the three experiments, was selected, and utilized for successive steps.

The Figure 7 shows a response from a positive pool of mRNA made in vitro from a cDNA pool of 2,000 plaque forming units. Unexpectedly, the reproducibility of the expression, has not improved with isolation. This indicates that a falsely positive signal was chosen, or some improvement was needed to increase the expression.

Changes in the strategy for clone isolation:

Several innovations were made regarding the cloning isolation technique. The small scale lambda cDNA preparation and the mRNA in vitro transcription used in the experiments of clone isolation were not found completely adequate. These methods did not give enough material for cDNA and mRNA controls and repeated injections of mRNA from a single preparation. The T3 promoter was also found not very efficient in using cDNA as a template in presence of the large lambda DNA molecule, possibly because of aspecific absorption. Hence, technical changes which are currently used include: 1) Preparation of large scale lambda DNA (50-100 ug each) from library pools. This method, although time consuming, is necessary for running controls on the quality of the cDNA used as a template. 2) Amplification by PCR of the cloning insert with relevant promoter. This step might be necessary and increases the ratio
of specific template (region containing the inserts), as compared to the lambda DNA and to improve mRNA in vitro synthesis (not shown). 3) Excision of the plasmid from the lambda. Plasmid was excised from 14 pools of 100,000 clones of the cDNA library, by in vivo excision through the "massive excision" protocol (Stratagene, California). Plasmid DNA has been prepared from each pool through a plasmid preparation (resulting in 50-100 ug of plasmid DNA from each) and is now available for mRNA synthesis. 10 ug of each prep were cut by XHO I and are currently being used in mRNA synthesis. This procedure will increase the promoter binding (required for mRNA synthesis) and efficiency of the in vitro transcription reaction, because of the less aspecific site on plasmid DNA respect to lambda. The PCR technique above described can be also applied more efficiently to the plasmid DNA if necessary. 3) Utilization of Megascript techniques (Ambion, Massachusetts) which make possible large amount of mRNA synthesis and therefore yield enough material to repeat injection of the same mRNA preparation.

These innovations are all aimed at improving the control steps in experiments where DNA is isolated and mRNA is made in vitro. Under these conditions, the expression cloning may be compared to the expression obtained with tissue isolated mRNA.

Additional strategies have been considered and might be used as an alternative. They include the possibility to engineer in the mRNA (by insertion in the vector) promoters specific to oocytes, with the aim of increasing the translation of mRNA injected (Krieg and Melton, 1984).
New libraries might be made and tested if existing libraries fail to show reproducible expression. The new libraries will be directly inserted in plasmids since the in vivo excision protocol mentioned above might reduce representation of the library.

Finally, a separate G-protein linked UTP "Nucleotide" receptor has been cloned in a different laboratory from a neuroblastoma cell line (Lustig et al., 1993). It is unlikely that this ATP receptor is similar to the one we are attempting to isolate since UTP does not elicit any receptor-mediated response in myocytes and because a membrane spanning domain as the one reported is unlikely to exist in a receptor activating cation conductances. However, since the characteristics of the cardiac receptor(s) are not known (Chapter 1). The sequence of the cloned UTP receptor might be useful for the isolation of cardiac receptors by PCR or probe analysis of available cardiac libraries.
CONCLUSIONS

In this chapter an expression cloning approach to the isolation of
the cardiac ATP receptor which induces a cation current in ventricular
myocytes is presented. In the next chapter this receptor and the one
inducing Na/Pi transport are characterized by photoaffinity labelling. The
results obtained with either methods are not inconsistent. The mRNA size
of both, the receptor-activated cation channel and Na-Pi transporter, is
consistent with the size of the proteins labelled as shown in the following
chapter. As the size of the two receptors appears similar, some
overlapping in the mRNA fractionation may be present.

Positive expression of ATP induced calcium transient in oocyte
injected with cardiac mRNA in luminescence studies was dependent on
the presence of extracellular calcium. This dependency is an evidence
against the indication of a major participation of intracellular calcium
release in the signal detected. The size fractionation studies are another
evidence against the need of the expression of the cardiac calcium-
induced calcium-release receptor, which is coded by larger mRNA and
was not included in the positive fraction.
Figure 1. A model representing ATP regulation of calcium channels in ventricular cells. Top left side shows activation of ATP dependent cation influx and voltage dependent calcium channels. Top right side shows ATP dependent activation of Na/Pi transport and Na/Ca exchange. The nature of the receptor coupling to these mechanisms is not known. Bottom figure shows calcium induced calcium release from the sarcoplasmic reticulum, which can be activated by the calcium influx.
ATP Receptor Modulation of Cell Calcium

Figure 1
Figure 2. 2A. Schematic representation of the procedure of mRNA isolation from rat heart and Xenopus Laevis oocytes injection. Details in the text. 2B. Representation of the photoluminescence assay. Aequorin was injected 3-5 hrs. before testing (50 nl of 1-2 mg/ml solution). Oocytes were tested singularly. The test was started by addition of ATP (or ATP analogues) in the cuvette and followed by addition of ionomycin. Calcium-dependent luminescence was detected by a photomultiplier: "Dual channel ratio fluorometer" (HV: 990. Full scale amplification) (Pennsylvania Biomedical-instrumentation, Philadelphia). Signal was recorded on a Linseis paper recorder.
2A. mRNA injection in oocytes

Rat Heart Ventricle Procurement → Tissue Lysis in Guanidinium Buffer → Phenol-Chloroform Extraction, Alcohol Precipitation → Female Xenopus Oocyte Ligar

Oocyte Defolliculation and Conditioning → Injection of 50-100 ng mRNA, subsequent aequorin injection

2B. ATP:Aequorin assay

1 ATP-Mg 2+ 5mM or Ionomycin

15 mM CaCl₂
20 mM Hepes pH 7.5
1 mM MgCl₂

Photomultiplier Detection of Luminiscence

Figure 2
Figure 3. Luminescence response to intracellular calcium transients. 3A. Water injected cells, subsequently injected with aequorin. These oocytes are unresponsive to either substance-K (50 nM) or MgATP (buffered to pH 7.2) (1 mM ea. addition). Extracellular solution is as indicated in figure 2B. The calcium ionophore ionomycin (4.6 ug/ml) induces a large increase in luminescence. 3B. Representative trace from oocytes injected with fmoles of mRNA coding for substance-K receptor. Addition of 50 nM substance-K induces a transient increase in luminescence. Extracellular solution is as shown in the previous figure, however this increase is dependent on intracellular calcium release, since is similar in absence of extracellular calcium (not shown). ATP did not induce a signal in these cells (not shown). 3C. Luminescence induced by extracellular ATP in single oocytes injected with total cardiac mRNA and aequorin. The increase in calcium depend on the presence of extracellular calcium. Reproducibility of the ATP response is discussed in the text. GTP or UTP (not shown) 1 mM do not induce a similar response.
Aequorin Responses to Intracellular Calcium Change

Figure 3
Figure 4. Figure 4A. Example of a sucrose-gradient fractionation of cardiac mRNA. In this case 27 fractions of 0.4 ml each were collected after spinning 34,000xg 20hrs at 4°C. The fractions were pooled for functional expression assays in pools of three fractions. Functional assays were done as in figure 3C (about 10 cells per pool or fraction, ea. test). (n5 different preparations tested 3-4 times each). Reproducibility is discussed in the text. The result of the pools injection are shown in figure 4B. Pool number three had peak ATP receptor expression. Subsequent injection of the fractions relevant to pools 2, 3 and 4, resulted in peak expression for fractions 8 and 9 (of pool 3). Fractions 4 and 12 (peripheral of pools 2 and 4) did not express ATP receptors (not shown).
Rat Heart mRNA Sucrose Fractionation

Figure 4
Figure 5. Flow chart representing cDNA synthesis and insertion in lambda-Unizap vector. The different enzymatic reaction conditions of first and second strand synthesis and the DEAE intercept method are reported in Table I. Other enzymatic reactions are as recommended by the manufacturers of the enzyme product. (Reverse transcriptase, RNase H, E.Coli ligase, Dna polymerase I, from BRL, Maryland; T4 Dna Polymerase, from Amersham, Illinois; T4 ligase, T4 Kinase from Stratagene, California). The flow-chart is self explanatory.
Flow chart for Zap-cDNA synthesis

First strand synthesis with methyl nucleotides
mRNA 5' → AAAAAA 3'
First strand DNA 3' ← TTTTTT Xhol Spnl 5'
Reverse Transcriptase

Second strand cDNA synthesis
5' → AAAAAA → 3'
3' → TTTTTT Xhol Spnl 5'
Rnase H, E.Coli Ligase, DNA Polymerase I

Blunting of double stranded cDNA
5' → Xhol Spnl 5'
3' → Ty DNA Polymerase

Ligate adaptors
Spnl adaptors
5' → Xhol Spnl 5'
Phospho (EcoR) Propylation

Heat inactivate the Ligase

Heat inactivate the Kinase

Kinase adaptors
Ty polynucleotide kinase

Xhol digest

Size selection of the cDNA by gel separation and DNA intercept on DEAE paper
EcoRI
5' → Xhol
1 to 9 kb insert

Directional insertion of the cDNA in the Unisap vector

Figure 5
Figure 6. Flow-chart representing ATP receptor clone isolation. PFU: plaque forming units, is the measure of number of clones in a pool as titered by bacterial infection. Fractions (e.g. 1-20 of 50,000 clones each were combined in pools e.g. I-V). For each pool mRNA was made in vitro from lambda preparations, and about ten cells were tested each assay. The test of the pools was repeated 3 times. The fractions of the most positive pool (judged from percent of cells in the three experiments) were then tested in the same way. The one considered more positive was subcloned in smaller pools.
Flow-chart of ATP receptor clone isolation attempt

Figure 6
Figure 7. ATP receptor expression from mRNA synthesized in vitro from a pool of 2,000 cDNA clones. Assay conditions as in figure 3C.
ATP Receptor Expression from cDNA Library

Figure 7
Table I. Schematic representation of cDNA first strand synthesis, cDNA second strand synthesis, and DEAE cDNA size separation by intercept. These methods were developed by modification of standard methods (Sambrook et al., 1989) for library synthesis.
Table I

cDNA first strand synthesis

For 50 ul:
X mRNA (5-10 ug)
X H2O DEPC treated
5ul 10X Buffer (.5M Tris,.75M KCl,.03M MgCl,.100 DTT)
In some experiments BRL first strand buffer was used
3ul 10 mM First Strand Methyl nucleotide mixture. The methylated is required to avoid cDNA cutting in successive steps
2ul Linker-Primer 1.4 ug/ul(Strategene,CA) containing the XHOI site
1ul RNase Block II (Strategene,CA)

X RNaseH+ Reverse Transcriptase (Superscript) BRL 200U/ul 200U/ug mRNA.
In some cases X AMV RT Boehringer 10U/ul

cDNA second strand synthesis:

As indicated in BRL protocol for second strand, plus the second strand nucleotide mixture from Strategene, to equilibrate the methyl nucleotide steps in the previous reaction. Optional alpha-32P-ATP in tracer amount was used as a marker in successive control gels, not in reactions in which the tracer method was used.

DEAE Cellulose DNA intercept method

I. Membrane Preparation (SS NAAS)
   A. Untreated or H2O treated membrane 7ug/cm² binding capacity (preferred for small amounts)
   B. Treated (as in Mannari) capacity increased up to 3 times but very inefficient removal

II. DNA Agarose Gel electrophoresis
   A. DNA gel as usual, 1/4 length
   B. Light staining in Ethidium Bromide
   C. Visualize with minimal exposure to UV lamp
   D. Incision and insertion of membranes below and above band of interest
   E. Run gel 2X voltage until all the band is adsorbed (membrane will glow orange)
   F. Remove membrane clean in H2O and place in elution buffer (the membrane has to be wet all the time)

III. DNA elution from membranes
   A. Elution with NaCl 1M, Arginine base 50 mM
      1. Two changes of 200 ul for each membrane
      2. Incubation at 68C 30' each, with occasional mixing
   B. Phenol-Chloroform extract and precipitation in ethanol Times 3
CHAPTER 4

CHARACTERIZATION OF MYOCARDIAL EXTRACELLULAR ATP RECEPTORS BY PHOTOAFFINITY LABELLING AND FUNCTIONAL ASSAYS
ABSTRACT

Extracellular ATP receptors in rat ventricular myocytes were investigated through intact cell photolabelling followed by protein isolation. 8-azido ATP (8Az-ATP) was used for labelling under specific conditions determined by parallel functional studies. In those studies ATP-induced cytosolic Ca$^{2+}$ transients were irreversibly and specifically inhibited by UV-photolyzed 8Az-ATP, but not by 2-azido ATP (2Az-ATP), even in the presence of high concentrations of phosphonucleotides not affecting myocardial ATP receptors.

This part of the study functionally identifies a new antagonist for two cardiac ATP receptor types previously recognized in our laboratory (De Young and Scarpa, 1991). In addition, the antagonist activity is retained in presence of millimolar UTP, a condition that increases tremendously its specificity. Finally, this antagonist has photolabelling activity. Because of this property one can use the antagonist to label intact cells, and the binding will resist further purification. Under those conditions background labelling is minimized and radioactive 8Az-ATP specifically labels a band of 45-48 kD on a SDS gel.

Labelling under the above conditions in presence of ATPcS or 2-methylthio-ATP (2-meSATP), which are selective for the two functionally different cardiac ATP receptors, shows two different proteins with similar molecular weight consistent with the possible labelling of these two receptors. These results confirm the findings of expression cloning.
INTRODUCTION

The exposure of many cell types to micromolar concentrations of extracellular ATP results in signals modulating several intracellular functions. Following the original evidence that extracellular ATP serves as a neurotransmitter (Burnstock, 1972, 1991), the spectrum of activities elicited by ATP has significantly expanded and now includes regulation of most parenchymal and epithelial cells (Eds. Dubyak and Fedan, 1990). This resulted in the classification of P$_2$-purinergic receptors which are ATP specific as compared to adenosine-specific P$_1$-purinergic receptors (reviewed by Kennedy, 1990).

Recently, several P$_2$ receptor subtypes in various cells and within the same cell type have been classified on the basis of functional responses and agonist specificity. Accordingly, evidence has been provided for receptors linked to G-proteins, activating channels or forming pores (for review see Gordon, 1986 and Dubyak, 1991). Heretofore, no receptor has been isolated and no information exists on even partial primary structure, a condition which confounds classification of ATP receptors and limits our knowledge of ATP interaction with the receptor(s) and the resulting cellular signals.

Previous studies from our laboratory (De Young and Scarpa, 1987, 1989) and others (Christie et al., 1992) have shown that extracellular ATP regulates transmembrane potential and cytosolic Ca$^{2+}$ in ventricular myocytes. At least two types of ATP receptors described in these cells
have been shown to be functionally distinct from ATP receptors in other cells. The first type has stringent requirements for ATP\textsubscript{Mg}, and responds to 2-meSATP and ATP, but not to ATP\textsubscript{S}, UTP, GTP, ADP or adenosine. The major effect of ATP\textsubscript{Mg} binding to this receptor is the activation of an inward non-selective cation depolarizing current. This event, which is not G-protein linked, is followed by the activation of voltage-sensitive Ca\textsuperscript{2+} channels, increase in Ca\textsuperscript{2+} influx and Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (De Young and Scarpa, 1987). The second type is activated by ATP or ATP\textsubscript{S}, but not 2-meSATP, probably requires G-proteins, and initiates uptake of both Pi and Na\textsuperscript{+} from the extracellular spaces (De Young and Scarpa, 1992).

Recently Boyers et al., 1991 labelled a P\textsubscript{2Y} receptor by using \( [\text{a}^{32}\text{P}] \)(Bz-ATP) in turkey erythrocytes membranes. This analogue labelled a protein of Mr 53,000. In the present study we have attempted to characterize myocyte ATP receptors through the use of photoaffinity labelling. We took advantage of the high specificity of nucleotides for these receptors and searched through functional assays for ATP analogues which could photolabel these receptors under conditions where unrelated ATP binding sites were saturated by large concentrations of nucleotides that did not affect ATP receptors.

Labelling with 8Az-ATP under these conditions identified a major protein band. This band may consist of two distinct proteins which are tentatively identified as the ATP binding sites for the two receptors activating cation conductance or Na/Pi cotransport.
METHODS

Cell Preparation

Ventricular myocytes were prepared from the hearts of Sprague-Dawley adult male rats as previously described (De Young, Giannattasio and Scarpa, 1989). The suspension of isolated myocytes was enriched for intact, rod-shaped myocytes by centrifugation on a Percoll step gradient: 3.7 ml of Joklik (Sigma Chemical, St. Louis, MO) media with 1% BSA was layered on 10 ml of Joklik media made with 60% Percoll in a sterile conical tube. Cells from a single rat heart were suspended in 20 ml of Joklik (1% BSA) and an aliquots of 10 ml were layered on parallel gradients. The gradients were centrifuged for 2-3 min in a table top centrifuge (approximately 300xg). The sediment was washed briefly in Joklik media, resedimented, and resuspended in low-phosphate Geigy buffer. The sedimented cells typically were 70% rod-shaped myocytes without other identifiable cell types. These cells are normally quiescent and the rate of spontaneous beating is less than one per minute. Yields of cells using this procedure were 2-3 x 10^6 cells/heart.

Intracellular Ca^{2+} Measurements

Loading with fura2/AM, (Molecular Probes), Eugene, OR) was done as previously described (De Young, Giannattasio and Scarpa,
1989). After Percoll enrichment, the cells were suspended in 15-20 ml Geigy buffer. Aliquots of 1 ml were assayed at 37°C with magnetic stirring in a custom-built fluorimeter. A 75 watt xenon lamp filtered at 340 nm served as the excitation source; fluorescence emission was collected after filtering through a 490 nm interference filter and measured at 90° from the source lamp by a photomultiplier tube. Changes in fluorescence intensity were plotted on a strip chart recorder.

As previously noted (De Young and Scarpa, 1989), precise calibration of the signal in terms of intracellular free calcium is difficult in myocytes. Hence, we used the ratio of the ligand-generated signal to that obtained by cell depolarization with KCl (30 mM) to standardize different cell samples.

*Labelling protocol*

8-azido-ATP (8Az-ATP) solution was prepared from cold and radioactive stock; typically 10 ul of nonradioactive 8Az-ATP (5mM) was added to 80 ul 8Az-ATP [α32P] (ICN: 160 uM, 15.2 Ci/mmol) to give a working solution of 700 uM (2.1 uCi/ul, 3.2 Ci/mmol) and was stored in the dark.

Cell aliquots (1 ml) were stirred in plastic cuvettes; in the controls 2-5 mM ATP was added prior to 8Az-ATP addition, whereas 300-800 uM UTP was added to the experimental samples. Some samples contained UTP plus either 2-meSATP or ATPγS (200uM). 8Az-ATP (10-15 ul of the
working solution; final concentration 7-10 uM) was rapidly mixed with the samples and photolyzed under a UV light (A+B) for 45-60 sec. Cells were sedimented through oil (dibutyl-phthalate: dioctyl-phthalate 2:1 by vol) and resuspended in Geigy buffer supplemented with 5 mM ATP. Cells were then sonicated for 45-60 sec with a Branson Sonifier model 185, using the microtip under maximum cavitation. The sample was briefly sedimented at 400xg, and the supernatant frozen in liquid nitrogen and lyophilized overnight. The pellet was discarded. The samples were boiled in SDS loading buffer with dithiothreitol and run on 7.5% or 11% polyacrylamide gels.

Gel scanning was executed using a USB (United States Biochemical) SciScan Tm 5000 optical scanner, and analysis was done using Bioanalysis software version 1.0.
RESULTS AND DISCUSSION

The addition of micromolar concentrations of extracellular ATP to isolated nonbeating ventricular myocytes activates Ca$^{2+}$ transients (De Young and Scarpa, 1987). Fig. 1A shows that the addition of ATP to fura2-loaded myocytes in physiological concentration of sodium and phosphate generates a Ca$^{2+}$ transient measured as an increase in fluorescence. Consistent with results previously seen (De young and Scarpa, 1987) a second addition of ATP results in a smaller transients. Depolarization of the cells by KCl produces further increase in cytosolic Ca$^{2+}$ as a result of calcium influx through voltage dependent channels. We used KCl as an internal control in the evaluation of the ATP response that is expressed as D ligand/D KCl ratio. Fig. 1A also shows that, in the absence of photolysis, 8Az-ATP, in micromolar concentrations, acts neither as an agonist nor as an antagonist with respect to the ATP response. Additionally, the figure shows that pre-exposure with UV light (A+B) at the energy used for photolabelling has no effect on the transients of Ca$^{2+}$ elicited by ATP or KCl. In fact, the response to added ATP was identical with or without previous UV light exposure (not shown). UV light irradiation in the presence of 8Az-ATP results in a nearly complete inhibition of the increase in Ca$^{2+}$ induced by ATP but not by KCl. The detailed mechanism by which 8Az-ATP inhibits the receptor only after UV irradiation is presently unclear. It is possible that the inhibition is a consequence of receptor crosslinking upon photolysis of the arylazide
and/or the inactivation or denaturation of receptors by the chemistry during photolabelling. In general, the efficiency of this reaction is low 2-3% (Dr. Ikebe, personal communication). From the gel shown later, it is apparent that a major protein band is labelled under those conditions and that this labelling is prevented by specific ligands of the receptors (ATP-S, 2-meS ATP or ATP). Hence, possible explanations are: a) that 8Az-ATP binds the receptor but with much lower affinity of ATP in absence of UV light; b) that there is an unusually high labelling efficiency; c) that an unknown percentage of the receptors is labelled and the remaining fraction is inactivated by chemical reactions during labelling. The alternative explanation that photolysis generates a product which, being a strong agonist, desensitizes the ATP response is not supported by the data of Fig. 1B, which shows that UV irradiation of 8Az-ATP does not itself produce an intracellular Ca$^{2+}$ increase. Furthermore, the addition of the azide pre-photolyzed in the absence of cells, results in no Ca$^{2+}$ increase and the successive addition of ATP produces a normal or slightly lower response (not shown).

Fig. 2 shows that the 8Az-ATP inhibition is maintained in the presence of large amounts of other phosphonucleotides. Concentrations of 200 uM ADP (Fig. 2A) or UTP up to 800 uM (Fig. 2B) do not interfere with the 8Az-ATP inhibition. The inhibition by 8Az-ATP is consistent with the agonist selectivity of the cardiac ATP receptors: UTP and ADP do not interfere with the ATP response nor with the ability of photolyzed 8Az-ATP to inhibit the response. These high concentrations of UTP might bind to
and saturate other ATP membrane binding sites and increase the labelling
specificity of cardiac ATP receptors by 8Az-ATP (see below). The obvious
experiment of competing 8Az-ATP with high concentrations of ATP in the
fura2 studies was unfeasible because high ATP concentrations result in a
long-lasting desensitization of the ATP dependent Ca\(^{2+}\) transient.

The specificities of photoaffinity analogues are shown in Table 1: the action of various analogues as either agonists or antagonists, with or
without UV light exposure is expressed relative to the ATP effect as a
pattern from several experiments. It is interesting to note that Bz-ATP
which has previously been shown to label G-protein coupled P\(_{2Y}\)
receptors (Boyer et al., 1991), does not interfere with the ATP response.
Even more striking is the finding that the 2Az-ATP, an analogue in Ng-C\(_{1}'\)
anti configuration which has been shown to label ATPase sites (Davis et
al., 1991), has no activity. Non-photolyzed 8Az-ATP, an analogue in Ng-
C\(_{1}'\) syn configuration is a very weak agonist but becomes a strong
antagonist after UV light exposure. This is an additional strong indication
of the substrate specificity of this receptors which peculiarly prefer
substrates in the Ng-C\(_{1}'\) syn configuration rather than the usual Ng-C\(_{1}'\)
anti configuration.

Fig. 3 shows a representative experiment in which 7 uM radioactive
8Az-ATP in the presence of 700 uM UTP was added and photolyzed in
freshly isolated intact myocytes. Upon cell filtration through oil and
sonication and solubilization of their membrane a large band calculated
from standards as near 48 kD and a less pronounced band of 90 kD (lane
1) are labelled. All the labelling is eliminated if 2-5 mM cold ATP was present before 8Az-ATP photolysis (not shown). Labelling of the 48 kD band (lane 1) is partially inhibited by either 2-meSATP or ATPγS (200uM) lane 2 and 3, and a shift of the labelled protein band is observed: 2-meSATP inhibits labelling of the lower portion of the band (lane 2), while ATPγS removes the upper portion. This is better seen from the scanning densitometry of the same gel (Fig. 3b), which indicates that the band of 48 kD is the result of the comigration of two distinct proteins. The selectivity with which 2-meSATP and ATPγS compete with the labelling corresponds to the strict agonist specificity of two previously reported separate myocardial ATP receptors (10). This conclusion is more quantitatively supported by the finding shown in Table 2 which shows that the sum of integrated optical densities (IOD) of the 2-meSATP and the ATPγS bands subtracted of the specific background, is close to the total IOD of the 48 kD total band. Since the labelling conditions in lane 1,3 are identical, except for the specific competitor in lanes 2 and 3, the results suggest that the competitors selectively eliminate portions of the band in lane 1, as already hinted by the slight difference in gel position observed in the linear scanning of Fig. 3.

In conclusion ATP-induced calcium transients in myocytes are irreversibly and specifically inhibited by UV photolyzed 8Az-ATP, even in the presence of high concentrations of several phosphonucleotides which do not effect myocardial ATP receptors. At these concentrations labelling of unrelated ATP binding sites is minimized and radioactive 8Az-ATP
specifically labelled a large band of 45-48 kD and one faint band of 90 kD on autoradiographed SDS gels.

Labelling of the 45-48 kD band can be inhibited by 2-meS-ATP and ATP_{γ}S. These two analogues are selective substrates for the activation of a fast calcium transient or sodium and phosphate uptake respectively. We have previously shown that extracellular ATP activates in myocytes both a fast calcium transient and a slower sodium/phosphate cotransport (De Young and Scarpa, 1991). 2-meSATP, but not ATP_{γ}S, activates the first rapid phase of Ca^{2+} transient and has no effect on Na^{+}/Pi cotransport. By contrast the latter is activated ATP_{γ}S. Additional evidence for two distinct receptors are the preliminary data showing that two distinct mRNA fractions from rat hearts express either response in a Xenopus oocyte expression system (Giannattasio, Powers and Scarpa, 1991, 1992). Hence, this data are short of demonstrating but are consistent with the inference that the 45-48 kD band is composed of two distinct ATP binding proteins that are involved in the different pathways of ATP signalling cascades in myocardial cells.

Since the publication of these data (Giannattasio et al., 1992) there have been two more communications, indicating labelling of ATP receptors that induce influx of extracellular calcium. In one is presented the labelling of a 68 KD molecule, pharmacologically characterized as the P_{2X} receptor of rat vas deferens (Bo et al., 1992). In the other is reported labelling of a ATP receptor of 53 KD in PC12 cells (Majid et al., 1992). The higher molecular weight of the receptor in PC12 cells as compared to the
cardiac receptors, is in agreement with the results of the mRNA fractionation studies shown in the previous chapter.
Fig. 1. Measurement of cytosolic free calcium in fura2/am loaded myocytes. The experimental conditions were as described in methods. The concentrations of the reagents indicated were: ATP 20 μM, 8Az-ATP 10 μM, KCl 30 mM. UV light (A+B) was applied for 40-50 seconds before (Fig. 1A) or after (Fig. 1B) 8Az-ATP.
Fig 1

\[ \Delta \text{Ligand vs. } \Delta \text{KCl} \]

UV Post-flash

UV Pre-flash

1 min
Fig. 2. Lack of effect of ADP or UTP on the 8Az-ATP inhibition. The conditions were similar to that of Fig. 1 Where indicated, ADP was 200 uM (Fig. 2A), or UTP 700 uM (Fig. 2B).
Fig. 3. Fig. 3A. SDS-gel of myocyte proteins after 8Az-ATP labelling. Labelling in intact myocytes was carried out in: UTP 800 uM (lane 1), UTP plus 200 uM 2-meSATP (lane 2), UTP plus 200 uM ATPcS (lane 3). Fig. 3B is a linear scanning of the same gel. The part of the gel scanned is within brackets. Intensity is in arbitrary units.
# Table I

**Agonist/Antagonist Activity of ATP Photoaffinity Analogues**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Agonism</th>
<th>Pre-UV Antagonism</th>
<th>Post-UV Antagonism</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>+++</td>
<td>+ (Des.)</td>
<td>+ (Des.)</td>
</tr>
<tr>
<td>Bz-ATP</td>
<td>+/-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2Az-ATP</td>
<td>+/-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8Az-ATP</td>
<td>+/-</td>
<td>ND</td>
<td>+++</td>
</tr>
</tbody>
</table>

**ND**: Non detectable  
**Des.**: Desensitization

*Table I. The analogues were tested for calcium transients as shown in Fig. 1. ATP concentration was 20 μM; Bz-ATP, 2Az-ATP, 8Az-ATP were 15 μM.*


Table 2
Density Determination of Gel Bands by Optical Scanning

<table>
<thead>
<tr>
<th>Lane Number</th>
<th>IOD</th>
<th>IOD (Background subtracted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9785</td>
<td>5520</td>
</tr>
<tr>
<td>2</td>
<td>6734</td>
<td>3523</td>
</tr>
<tr>
<td>3</td>
<td>5927</td>
<td>2587</td>
</tr>
</tbody>
</table>

Table 2. Intensity numbers are in arbitrary units. IOD (integrated optical density) was calculated from the gel shown in Fig. 3 by integrating pixel density from equivalent areas at each lane encompassing the 45-55 kD bands. For each lane, the background was obtained by integrating the density in the area defined between the two arrows. This was then subtracted from the respective individual scans.
CHAPTER 5

CALCIUM CURRENTS IN THE A7r5 SMOOTH MUSCLE-DERIVED CELL LINE

Calcium-dependent and voltage-dependent inactivation
ABSTRACT

Inactivation of a dihydropyridine-sensitive calcium current was studied in a cell line (A7r5) derived from smooth muscle of the rat thoracic aorta. Inactivation is faster with extracellular Ca\(^{2+}\) than with Ba\(^{2+}\). In Ba\(^{2+}\), inactivation increases monotonically with depolarization. In Ca\(^{2+}\), inactivation is related to the amount of inward current, so that little inactivation is seen in Ca\(^{2+}\) for brief depolarizations approaching the reversal potential. Longer depolarizations in Ca\(^{2+}\) reveal two components of inactivation, the slower component behaving like that observed in Ba\(^{2+}\). Furthermore, lowering extracellular Ca\(^{2+}\) slows inactivation. These results are consistent with the coexistence of two inactivation processes, a slow voltage-dependent inactivation, and a more rapid current-dependent inactivation which is observable only with Ca\(^{2+}\). Ca\(^{2+}\)-dependent inactivation is decreased but not eliminated when intracellular Ca\(^{2+}\) is buffered by 10 mM BAPTA, suggesting that Ca\(^{2+}\) acts at a site on or near the channel. We also studied recovery from inactivation following either a short pulse (able to produce significant inactivation only in Ca\(^{2+}\)) or a long pulse (giving similar inactivation with either cation). Surprisingly, recovery from Ca\(^{2+}\)-dependent inactivation was voltage-dependent. This suggests that the pathways for recovery from inactivation are similar regardless of how inactivation was generated. We propose a model where Ca\(^{2+}\) - and voltage-dependent inactivation occur independently.
INTRODUCTION

Two primary mechanisms of inactivation have been described for calcium channels, voltage-dependent and Ca$^{2+}$-dependent inactivation (Eckert and Chad, 1984; Carbone and Swandulla, 1989). Ca$^{2+}$-dependent inactivation is greatly decreased when Ca$^{2+}$ is replaced by Ba$^{2+}$, and can often be prevented by buffering intracellular Ca$^{2+}$. Usually, Ca$^{2+}$-dependent inactivation is maximal near the voltage that generates peak inward current and inactivation decreases at more positive potentials, but this is neither necessary (Gutnick et al., 1989) nor sufficient (Jones and Marks, 1989) to demonstrate Ca$^{2+}$ dependence. Voltage- and Ca$^{2+}$-dependent inactivation mechanisms coexist in many cell types (Brown et al., 1981; Kass and Sanguinetti, 1984; Lee et al., 1985; Satin and Cook, 1989), including smooth muscle (Jmari et al., 1986; Ganiktevich et al., 1987).

Entry of Ca$^{2+}$ through dihydropyridine-sensitive calcium channels is critical for arterial smooth muscle tone (Nelson et al., 1990). Inactivation of calcium channels could play an important role in regulation of channel availability, but there is little information on the kinetics and mechanisms of calcium channel inactivation in such cells.

We demonstrate in A7r5 cells that two separable mechanisms of inactivation coexist, one dependent on membrane potential and the other on Ca$^{2+}$ entry. Ca$^{2+}$ appears to act locally to inactivate the channel through which it enters (Mazzanti and DeFelice, 1990; Yue et al., 1990),
not diffusely by increasing cytosolic Ca$^{2+}$ (Gutnick et al., 1989). We propose a model for calcium current inactivation that explains the major features of our results.

Preliminary reports of some of these results have appeared in abstract form (Giannattasio et al., 1989, 1990).
METHODS

Voltage clamp

A7r5 cells (Kimes and Brandt, 1976) were cultured as described (Marks et al., 1990). Currents were recorded in the whole-cell configuration using an Axopatch 1B (Axon Instruments, Foster City, CA) or List EPC 7 (List Medical, Darmstadt/Eberstadt, Germany) amplifier. We used electrodes with series resistances of 2-5 MΩ, as estimated from cancellation of the capacity transient. Series resistance errors should therefore have been < 5 mV for the < 1 nA currents studied here. Spherical cells with smooth surfaces, no evident membrane folding, and few visible surface attachments, were selected to reduce space clamp errors. Despite these precautions, some cells showed signs of slow voltage clamp, including large residual capacity transients following optimal compensation. Other cells appeared to be excellently clamped, with rapid (τ < 1 ms) tail currents (Obejero-Paz et al., 1990). In cells studied here, the currents induced by depolarizing steps were well controlled, with graded activation and no "notches."

pClamp software (version 5, Axon Instruments) was used with IBM AT-compatible microcomputers to both generate voltage commands and acquire data. Records were generally analog filtered at 5 kHz (depending on the sampling rate) and then digitally filtered during the analysis at 1 kHz. Analysis was also done with pClamp (Clamp and Clampfit programs). Leak subtraction was done either by adding the inverted and
scaled currents generated by 1/4 amplitude hyperpolarizing pulses, or using values from linear regression of currents generated by hyperpolarizing pulses. Further analysis was done with Lotus 1-2-3 and figures were prepared with Micrografx Draw Plus.

Solutions and materials

The standard extracellular solution contained (mM): NaCl 112.5, KCl 5, MgCl₂ 1.2, CaCl₂ or BaCl₂ 10, NaHEPES 2.5, and glucose 10, adjusted to pH 7.4 with NaOH. When necessary, NaCl was osmotically substituted for CaCl₂ or BaCl₂. The standard intracellular solution contained (mM): CsCl 120, MgCl₂ 4, TrisATP 5, CsEGTA 1, CsHEPES 2.5, adjusted to pH 7.2 with CsOH. Where noted, CsEGTA was varied from 0 to 10 mM, or substituted with 1-10 mM CsBAPTA, with changes in CsCl to maintain osmolarity. The extracellular solution was changed using a gravity flow system controlled remotely by solenoid valves.

EGTA [ethyleneglycol-bis-(b-aminoethyl ether) N,N,N',N'-tetraacetic acid], HEPES [N-(2-hydroxy-ethyl)piperazine-N'-(2-ethanesulfonic acid)] and ATP (Na or Tris salt) were purchased from Sigma (St. Louis, MO). BAPTA [1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid] was from Sigma (free acid) or from Molecular Probes (Eugene, OR) (cesium salt). Other chemicals were reagent grade.

Isolation and stability of calcium current
The predominant voltage-dependent current in A7r5 cells is a dihydropyridine-sensitive (L-type) calcium current (Marks et al., 1990). With intracellular CsCl, we did not observe any time-dependent outward current, even for > 300 ms steps to strongly depolarized voltages (+40 to +70 mV). Replacement of internal and/or external monovalent cations with N-methyl D-glucamine did not modify the current profile. In some cells a transient (T) current was present, but it was absent or < 10% of the total current in the cells reported here. The normal holding potential (-60 mV) was changed to -40 or -50 mV (as noted) when T-current had been observed recently.

The amplitude of current, and the extent of rundown with time, varied widely among cells. Many cells were rejected due to small (< 50 pA) initial currents. But in many cases, with internal MgATP and EGTA, calcium currents could be recorded for more than 1 hour. Rundown appeared to be faster without EGTA or BAPTA, and with long and frequent depolarizing pulses.

Computer modeling

The AXOVACS programs (Axon Instruments) were modified to simulate calcium currents. Other programs were written in QuickBASIC (Microsoft, Redmond, WA) to calculate properties of the models, using in part the analytic solution to the general three state cyclic model (Gutnick et al., 1989).
RESULTS

Inactivation

Inactivation is faster in Ca$^{2+}$ than in Ba$^{2+}$ (Figs. 1-2). Inactivation can be observed either as the time-dependent decrease of inward current during a maintained depolarization, or as a decrease of the peak current generated by a second test pulse in a double pulse experiment (Eckert and Tillotson, 1981). A 60 ms prepulse generates ~50% inactivation in Ca$^{2+}$ but only ~10% in Ba$^{2+}$ (Fig. 1). Maximal inactivation in Ca$^{2+}$ is observed with prepulses giving maximal inward current, with less inactivation at more positive voltages. Therefore, except where noted, inactivation was measured at the voltage producing peak inward current (usually +20 mV in 10 mM Ca$^{2+}$, and +10 mV in 1 mM Ca$^{2+}$ or 10 mM Ba$^{2+}$).

Longer prepulses (330 ms) in Ba$^{2+}$ produced 32 ± 4% inactivation (mean±SEM, n=14) (Fig. 2). The range was 22-50%, except for one cell that showed no inactivation (see McCarthy and Cohen, 1989). In Ba$^{2+}$, inactivation increases almost monotonically with voltage. The same pulse length with Ca$^{2+}$ produces maximal inactivation for prepulses giving maximal inward current, but a considerable amount of inactivation also occurs at more depolarized voltages. Inactivation was considerably slower in Ba$^{2+}$ than in Ca$^{2+}$ in 9 of 9 cells tested under both conditions.

The relation of current to inactivation is examined more directly, using normalized values, in Fig. 3. Inactivation closely parallels the
current for short prepulses in Ca$^{2+}$ (Fig. 3 A). In Ba$^{2+}$, inactivation parallels the increase in current at negative voltages, but strong depolarizations that do not generate large currents also reduce the postpulse (Fig. 3 C). For long prepulses in Ca$^{2+}$, the inactivation curve is broadened, with considerable inactivation at voltages producing little inward current (Fig. 3 B). The broadening is especially clear at more depolarized voltages, whether inactivation is compared to peak current or to the integral of Ca$^{2+}$ entry during the entire prepulse.

Inactivation measured by the postpulse agrees well with inactivation during the prepulse, for voltages where the prepulse current is large enough for its inactivation to be accurately measured (Fig. 3). This is additional evidence that the calcium current is well isolated, and that we are measuring inactivation rather than unrelated phenomena such as activation of an outward current. Thus, the time course of inactivation can be measured directly from the amplitude of the current during the prepulse (Fig. 4).

*Inactivation kinetics*

The time course of inactivation during a 60 ms pulse in Ca$^{2+}$ could be well fitted by a single exponential (Fig. 4 A). Similar rapid inactivation ($\tau = 15 - 45$ ms) was observed in 20 of 26 cells tested in Ca$^{2+}$. In contrast, a good fit to the time course of inactivation during a 330 ms pulse in Ca$^{2+}$ required the sum of two exponentials of similar amplitude (Fig. 4 C,D). In
Ba$^{2+}$ one exponential fit the time course of inactivation (Fig. 4 B), with time constant 210 ± 13 ms (n=20, range 120-343 ms).

The difference in inactivation rates allows selective generation of Ca$^{2+}$- and voltage-dependent inactivation: 60 ms pulses in Ca$^{2+}$ generate predominantly Ca$^{2+}$-mediated inactivation, whereas 330 ms pulses in Ba$^{2+}$ generate inactivation that appears to depend solely on voltage (Figs. 1-3). Fig. 5 plots time constants measured for each condition, at different voltages. The fast component in Ca$^{2+}$ is fastest at or near the voltage producing peak current, suggesting current dependence. In Ba$^{2+}$, a slight increase in inactivation rate occurs at more depolarized voltages. The faster time constant measured from 330 ms pulses in Ca$^{2+}$ is also dependent on current and is slightly faster (5-10 ms) than the single time constant fit to short pulses. The slower time constant in Ca$^{2+}$ is more variable, and two time constants are not always well resolved at more depolarized voltages.

*Dependence on intracellular calcium*

The preceding results suggest the coexistence of two inactivation processes, one fast (~20 ms) and dependent on Ca$^{2+}$ entry, one slower (~200 ms) and voltage-dependent. We tested this hypothesis with procedures designed to modulate the Ca$^{2+}$-dependent component: changing the level of intracellular Ca$^{2+}$ buffer, and decreasing the current with low [Ca$^{2+}$]o.
Inactivation was similar with 1 mM or 10 mM EGTA, or with no added calcium buffer. Inactivation rates in Ca\(^{2+}\) with 10 mM BAPTA were generally slower than with 1 mM EGTA, but clearly faster than with Ba\(^{2+}\) (Fig. 6). From fits with two exponentials, the faster time constant for inactivation was 21 ± 2 ms (n=3) with 1 mM EGTA, and 40 ± 3 ms (n=3) with 10 mM BAPTA, in one batch of cells. Single exponential fits to inactivation during 60 ms pulses gave time constants of 41 ± 3 ms (n=8) with BAPTA. Ca\(^{2+}\)-dependent inactivation could still be observed with 10 mM BAPTA in cells held for several minutes with low series resistance, where the cell is likely to be well dialyzed (Fig. 6). We conclude that strong buffering of cytoplasmic Ca\(^{2+}\) does not eliminate Ca\(^{2+}\)-dependent inactivation.

Ca\(^{2+}\)-dependent inactivation still occurs after rundown of the whole-cell current to 20% of its initial value (Fig. 6), which should greatly reduce inactivation resulting from buildup of cytoplasmic Ca\(^{2+}\). In 6 cells (with 1 mM or 10 mM EGTA) where the peak current ran down by 46 ± 6%, time constants for inactivation during 60 ms pulses were 23 ± 2 ms initially and 22 ± 3 ms after rundown.

Parallel decreases of the whole cell calcium current and rate of inactivation were observed upon changing extracellular Ca\(^{2+}\) from 10 mM to 1 mM. (Fig. 7). In 5 cells tested at both concentrations, time constants for inactivation were 23 ± 2 ms in 10 mM Ca\(^{2+}\) and 58 ± 6 ms in 1 mM Ca\(^{2+}\). These effects were reversible, and thus not secondary to rundown (not shown). Longer prepulses in 1 mM Ca\(^{2+}\) produce more inactivation,
as in 10 mM Ca\(^{2+}\) or 10 mM Ba\(^{2+}\). Preliminary results show that inactivation is not changed by decreasing \([\text{Ba}^{2+}]_o\) from 10 mM to 1 mM.

**Recovery from inactivation**

It might be supposed that recovery from Ca\(^{2+}\)-dependent inactivation would require removal of intracellular Ca\(^{2+}\) following the end of the depolarization. This would predict that recovery from Ca\(^{2+}\)-dependent inactivation and recovery from voltage-dependent inactivation should differ qualitatively and quantitatively.

We examined the voltage- and ion-dependence of recovery from inactivation with the protocol of Fig. 8. For long pulses in Ca\(^{2+}\), recovery is faster at more hyperpolarized potentials. Voltage steps directly to -30 or -90 mV do not markedly inactivate or facilitate the current. Fig. 9 compares results in three conditions: short prepulses in Ca\(^{2+}\), long prepulses in Ca\(^{2+}\) and long prepulses in Ba\(^{2+}\). Recovery from inactivation is voltage dependent, even for short pulses in Ca\(^{2+}\) where development of inactivation is Ca\(^{2+}\)-dependent.

Inactivation and recovery at -30 mV are examined more closely in Fig. 10. On these slower time scales, some degree of inactivation is apparent. The amount of inactivation at -30 mV varies among cells, probably since that voltage is near the threshold for inactivation, where slight voltage shifts would have substantial effects. In this cell, inactivation and recovery protocols converge in ~1 s following brief prepulses in
Ca$^{2+}$ (Fig. 10A), whereas $>2$ s are required following long pulses (Fig. 10B,C).

Time constants for recovery from inactivation were measured from the protocols of Figs. 8-10 (Table 1). At -30 mV, recovery is faster following a short prepulse in Ca$^{2+}$ than following longer pulses. The time constant of recovery is smaller at negative voltages, and the time constants at -90 mV are similar under all three conditions. We conclude that recovery from inactivation is voltage dependent, both following Ca$^{2+}$-dependent inactivation and following voltage-dependent inactivation.

Model

The voltage-dependent inactivation seen in Ba$^{2+}$ can be described by a three-state sequential model with closed, open, and inactivated states:

\[
\begin{align*}
C & \underset{k_1}{\xrightarrow{k_2}} O & \underset{I_V}{\xleftarrow{k_{-1}} O} & \underset{k_{-2}}{\xrightarrow{k_2}} C
\end{align*}
\]

(Scheme 1)

Activation kinetics were approximated with rate constants depending symmetrically on voltage, with voltage dependence chosen to reproduce the observed current-voltage curve:
\[ k_1 = 200 \, e^{0.06(V-5)}; \, k_{-1} = 200 \, e^{-0.06(V-5)} \]

(1,2)

with units s\(^{-1}\) and mV. Since inactivation in Ba\(^{2+}\) follows activation of the current at negative voltages, microscopic inactivation need not depend on voltage, as macroscopic inactivation would be driven by the voltage dependence of activation. This would also explain the relative voltage insensitivity of the inactivation rate in Ba\(^{2+}\) (Fig. 5). But for recovery from inactivation to be more rapid with hyperpolarization, the transition from the inactivated to the open state must be voltage-dependent. For

\[ k_2 = 4; \, k_{-2} = 0.5 \, e^{-0.03(V-5)} \]

(3,4)

the voltage dependence of recovery from inactivation in Ba\(^{2+}\) (Table 1) is fit reasonably well.

Since Ca\(^{2+}\)-dependent inactivation survives strong Ca\(^{2+}\) buffering (Fig. 6), we assume that Ca\(^{2+}\) acts locally on the same channel through which it enters (see Discussion). This has the practical advantage of eliminating the need for detailed modeling of Ca\(^{2+}\) diffusion and buffering in cytoplasm. We use the Goldman-Hodgkin-Katz current equation to approximate the voltage dependence of Ca\(^{2+}\) influx through an open channel (neglecting efflux), and we incorporate saturation of the channel by multiplying by a mass action factor:
\[ G = f \nu/(1 - e^{-\nu}), \]

where \( f = [Ca^{2+}]_0 / ([Ca^{2+}]_0 + K) \), \( K = 2 \) mM, \( \nu = 2 \cdot V/25 \), and the single-channel calcium current is proportional to \( G \). Inactivation and recovery in \( Ca^{2+} \) can now be described by a model where \( Ca^{2+} \)- and voltage-dependent inactivation proceed independently:
How was this model chosen? The existence of two distinct time constants for inactivation in Ca\textsuperscript{2+} suggests a separate Ca\textsuperscript{2+}-dependent inactivated state, rather than an action of Ca\textsuperscript{2+} to modulate the rates of voltage-dependent inactivation. This conclusion is greatly strengthened by the observation that intracellular trypsin selectively removes voltage-dependent inactivation (Obejero-Paz et al., 1991). Independent inactivation processes allow efficient movement of the initially Ca\textsuperscript{2+}-inactivated channels to a voltage-inactivated state, which makes recovery from inactivation similar following long depolarizations in Ba\textsuperscript{2+} and Ca\textsuperscript{2+} (Table 1). Since Ca\textsuperscript{2+}-dependent inactivation is allowed only from the open channel, the model does not obey microscopic reversibility; flux around the cycle is driven by Ca\textsuperscript{2+} entry.

For \( k_3 = 70 \cdot \text{G} \) and \( k_{-3} = 9 \), in 10 mM Ca\textsuperscript{2+}, steps < 100 ms to +20 mV produce predominantly Ca\textsuperscript{2+}-dependent inactivation, but the \( I_V \) state is favored for long pulses. These values are roughly equivalent to the
statement that every $10^5$th Ca$^{2+}$ ion that enters prevents current flow through the channel for 110 ms.

This model produces reasonable fits to the voltage dependence of inactivation and recovery in Ba$^{2+}$ and in Ca$^{2+}$ (Fig. 11, Table 1), if kinetics are shifted 10 mV toward depolarized voltages in Ca$^{2+}$. The model predicts that recovery in Ca$^{2+}$ should require two exponentials, especially following short pulses, but the present data on recovery from inactivation are not adequate to resolve multiple time constants. The model also predicts less current and less inactivation in low Ca$^{2+}$ (Fig. 7).

Scheme 2 differs considerably from previous models of calcium current inactivation. Separate Ca$^{2+}$-dependent and voltage-dependent inactivated states have been proposed previously (Yatani et al., 1983; Gutnick et al., 1989), but on those models Ca$^{2+}$- and voltage-dependent inactivation were mutually exclusive, not independent. Also, the model of Gutnick et al. (1989) places the site of Ca$^{2+}$ action at a distance from the channel. The surprising consequence is that Ca$^{2+}$-dependent inactivation depends not on Ca$^{2+}$ entry during the voltage step, but on the resting level of cytoplasmic Ca$^{2+}$. This predicts a monotonic dependence of Ca$^{2+}$-mediated inactivation on voltage, in agreement with their observations, but not with our data (Fig. 1) or most other data on calcium current inactivation.
DISCUSSION

Two components to inactivation

The rapid component of inactivation in Ca$^{2+}$ is dependent on Ca$^{2+}$ entry. The slow component in Ca$^{2+}$, and essentially all inactivation in Ba$^{2+}$, are voltage-dependent. Although similar conclusions have been drawn in other cases, the evidence here will be reviewed briefly.

Rapid inactivation is seen with Ca$^{2+}$ but not Ba$^{2+}$, parallels the amount of inward current, and is decreased at low [Ca$^{2+}$]. This is strong evidence for inactivation mediated by entry of Ca$^{2+}$.

The near monotonic increase in inactivation in Ba$^{2+}$ indicates voltage-dependent inactivation. However, the slight decrease in inactivation at positive voltages in Ba$^{2+}$ (Fig. 2) might suggest a contribution of current-dependent inactivation. For Ba$^{2+}$-dependent inactivation to be the *predominant* mechanism, voltage-dependent inactivation must be restricted to extreme positive voltages, since inactivation in Ba$^{2+}$ does not show a clear current-dependence. That would require a steep voltage-dependence for the microscopic inactivation process, despite the flat voltage-dependence of the inactivation time constant in Ba$^{2+}$ (Fig. 5). We find this much less plausible than our model, where macroscopic inactivation in Ba$^{2+}$ is driven by the voltage-dependence of the activation process.
Voltage-dependent inactivation also occurs with Ca\(^{2+}\), since long pulses in Ca\(^{2+}\) produce inactivation even at extreme positive potentials (Fig. 2), the time course of inactivation is fit by the sum of two exponentials (Fig. 4), and recovery from inactivation is similar following long pulses in Ca\(^{2+}\) and Ba\(^{2+}\) (Table 1). Some Ca\(^{2+}\)-dependent inactivation might occur at extreme positive potentials, as some Ca\(^{2+}\) entry is expected even positive to the reversal potential. However, we calculate that Ca\(^{2+}\) influx at +70 mV is < 8% of the peak current at +20 mV, either from the Goldman-Hodgkin-Katz current equation (Fig. 11), or from the Almers and McCleskey (1984) model for calcium channel permeation, assuming activation kinetics as in Eq. 1-2 above.

The site of Ca\(^{2+}\) action in Ca\(^{2+}\)-dependent inactivation

Gutnick et al. (1989) calculated that high concentrations of BAPTA buffer Ca\(^{2+}\) within microseconds further than a fraction of a micrometer from the channel. This suggests that Ca\(^{2+}\) either acts at relatively high concentration in the immediate vicinity of the channel (Chad and Eckert, 1984), or at low concentration throughout the cell (Gutnick et al., 1989).

Inactivation resulting from increases in bulk cytoplasmic Ca\(^{2+}\) should be prevented by intracellular EGTA or BAPTA, as found for invertebrate neurons (Eckert and Chad, 1984; Gutnick et al., 1989). However, this is less clear for calcium currents of vertebrate smooth muscle (Ganitkevich et al., 1987; Katzka and Morad, 1989). Our observation that BAPTA slows Ca\(^{2+}\)-dependent inactivation, but does not
eliminate it, suggests that Ca\(^{2+}\) acts at a site where BAPTA buffers Ca\(^{2+}\) transients only partially. This would place the site of action outside the channel pore (which should be inaccessible to BAPTA) but within \(-0.1\) \(\mu\)m of the channel (Gutnick et al., 1989). An obvious possibility is binding to a cytoplasmic domain of the channel protein.

Attempts to mimic inactivation by raising \([\text{Ca}^{2+}]_i\) have produced inconsistent results. Inhibition of calcium currents by \(-100\) nM \([\text{Ca}^{2+}]_i\) has been reported (Byerly and Moody, 1984; Dupont et al., 1986; Ohya et al., 1988), but higher concentrations were required in other studies (Plant et al., 1983; Gutnick et al., 1989). These discrepancies may result in part from difficulties in maintaining a defined \([\text{Ca}^{2+}]_i\) in dialyzed cells (Byerly and Moody, 1984). Release of "caged" Ca\(^{2+}\) at micromolar levels by photolysis of buffers rapidly (\(\tau \sim 7\) ms) but partially reduced calcium current in sensory neurons (Morad et al., 1988), but actually enhanced calcium current in cardiac cells (Gurney et al., 1989). Cardiac calcium channels in planar bilayers are rapidly blocked by Ca\(^{2+}\), with an apparent dissociation constant of 4 mM, and no change in gating kinetics (Rosenberg et al., 1988). These effects are difficult to relate to the normal inactivation process.

It has been proposed that Ca\(^{2+}\)-dependent inactivation results from a dephosphorylation reaction (Chad and Eckert, 1986; Kalman et al., 1988). However, the rapid rates of inactivation (\(-25\) ms) and recovery (\(-120\) ms) at room temperature are at the upper limit of the rates expected for phosphatase or kinase reactions. It is also possible that
enzymatic regulation of calcium channel availability does occur, but is distinct from the rapid inactivation process discussed here.

Mazzanti and DeFelice (1990) and Yue et al. (1990) recently reported Ca\(^{2+}\)-dependent inactivation at the single-channel level, with cell-attached patches on cardiac cells. A channel inactivates slowly with Ba\(^{2+}\), even when Ca\(^{2+}\) is entering through all of the other calcium channels in the cell (Mazzanti and DeFelice, 1990). This is conclusive evidence for local, channel-by-channel inactivation.

**Role of calcium channel inactivation**

Since intracellular Ca\(^{2+}\) is the primary signal for muscle contraction in cardiac and smooth muscle, Ca\(^{2+}\)-dependent inactivation could serve as a potent negative feedback mechanism for regulation of contraction. This is particularly true in arterial smooth muscle, where Ca\(^{2+}\) entry normally occurs in response to slow, graded depolarization rather than action potentials (Nelson et al., 1990). Given the strong driving force for Ca\(^{2+}\) in the critical voltage region (-40 to -55 mV), the large single-channel currents could produce strong Ca\(^{2+}\)-dependent inactivation on a channel-by-channel basis. Such voltages might also produce significant voltage-dependent inactivation, especially if slow inactivation occurs (Schouten and Morad, 1989).
Figure 1. Inactivation of calcium current generated by short depolarizations in A7r5 cells. 60 ms depolarizing prepulses of variable amplitude were given to induce inactivation, followed by a second constant pulse to the voltage giving maximal inward current (+10 mV in 10 mM Ba$^{2+}$ and +20 mV in 10 mM Ca$^{2+}$) to assay inactivation. At the left, leak-subtracted currents are shown (filtered at 5 kHz), for the records where the prepulse and postpulse voltages were equal. Current-voltage relations are shown at the right. Currents were measured at the peak current during the prepulse (△), at the end of the prepulse (▼), and at peak current during the test pulse (●). The current-voltage curves shown are averages of two protocols, with prepulse voltages given in ascending and descending order, to compensate for rundown. Rundown, measured as the decrease in peak current between the two runs, was < 3%.
Figure 2. Inactivation of calcium current generated by longer depolarizations. Protocols as in Fig. 1, except that prepulses lasted 330 ms, and records were filtered at 100 Hz. The data for Figs. 1-2 were recorded from the same cell, ~50 min (Ba$^{2+}$) and ~80 min (Ca$^{2+}$) after the start of whole-cell dialysis. Rundown was 11% in Ca$^{2+}$ and 35% in Ba$^{2+}$. 
Figure 3. Normalized currents and inactivation, from the data of Figs. 1-2. Currents (△) and current integrals (●) were normalized to values at peak inward current. Inactivation was measured in two ways, from the decline in current during the prepulse (▼) (current at the end of the pulse divided by the peak current at that voltage), and from the postpulse amplitude (●) (divided by the postpulse amplitude when no prepulse was given). The fractional inactivation measured in either way was then normalized to the fractional inactivation measured at the voltage generating maximal inward current. Inactivation during the prepulse was calculated only for voltages where the current was at least 20% of the maximum. The analysis for Ba$^{2+}$ after 60 ms prepulses is not shown since the amount of inactivation generated during that protocol was very small.
Fig 3

A. Ca\(^{2+}\), short

B. Ca\(^{2+}\), long

C. Ba\(^{2+}\), long

- ▼ Inactivation, pre
- ■ Inactivation, post
- ○ Current integral
- △ Peak current
Figure 4. The time course of inactivation. Currents (thick traces) were fitted to single exponentials (thin traces) during (A) a 60 ms depolarization to +20 mV in Ca\(^{2+}\) (\(\tau = 24.5\) ms), (B) a 330 ms depolarization to +10 mV in Ba\(^{2+}\) (\(\tau = 232\) ms), and (C) a 330 ms depolarization to +20 mV in Ca\(^{2+}\) (\(\tau = 50.2\) ms), from the same cell as Figs. 1-3. (D) A fit of the sum of two exponentials (\(\tau = 21.5\) ms, amplitude 0.15 nA; \(\tau = 139\) ms, amplitude 0.11 nA) to the data of part C. Dashed lines indicate zero current.
Figure 5. Inactivation time constants. Each symbol is a different cell. Values are from single exponential fits to currents during 330 ms steps (solid symbols, Ba$^{2+}$), or 60 ms steps (open symbols, Ca$^{2+}$). The arrows mark the voltage inducing peak inward current under each condition. Only a narrow voltage range could be examined, where the current was large enough and sufficient inactivation occurred. We found a $\sim 10$ mV variation in the position of peak current for different cells in the same conditions, but generally the fast time constant in Ca$^{2+}$ was the fastest at or slightly hyperpolarized to peak current, with slower values on both sides.
Fig 5
Figure 6. Effect of 10 mM intracellular BAPTA on calcium current inactivation. Currents (filtered 200 Hz) were from voltage steps to the point of peak inward current (+20 mV in Ca$^{2+}$, +10 mV in Ba$^{2+}$) from the holding potential of -50 mV, recorded at the times indicated. Currents were scaled to the peak current for each record.
Figure 7. Inactivation of calcium current generated by a short pulse in 1 mM [Ca^{2+}]_o, with the same protocol as Fig. 1.
Figure 8. Recovery from inactivation. At the left, the primary protocol used is illustrated for 360 ms prepulses to +20 mV in Ca²⁺. Following the prepulse, the cell was repolarized to -30, -60, or -90 mV for a variable time, and then a test pulse was given to +20 mV to test the amount of recovery. A similar protocol, shown at the right, was conducted to test for development or removal of inactivation by the steps to -30 or -90 mV. Design of the protocols for recovery from inactivation was complicated by several factors. (1) Inactivation was not complete, and the extent of inactivation measured during the prepulse could differ slightly from that measured at postpulses (due to inactivation during the postpulse). (2) When recovery was measured at voltages other than the holding potential, the maximal level of recovery was not necessarily the peak current during the prepulse. To correct for this, the maximal recovery level was defined as the point where recovery from inactivation and development of inactivation converge. That required the separate protocol to determine the time course of inactivation (or facilitation) at each voltage. (3) Rundown, which is exacerbated by long depolarizing steps, required that each current be normalized to the peak current during a prepulse. For the development of inactivation protocol, the prepulse had to be brief, and sufficient time had to be allowed for inactivation generated by the prepulse to recover.
Figure 9. Voltage dependence of recovery from inactivation in three different conditions. The time course of inactivation (open symbols and dashed lines) and recovery from inactivation (solid symbols and lines) are compared at each voltage, by the protocols of Fig. 8. Prepulses were 75 ms in Ca$^{2+}$ (A), 360 ms in Ca$^{2+}$ (B), or 360 ms in Ba$^{2+}$ (C). Values are peak currents during postpulses, normalized to the peak current during the prepulse (a/b; see inset). The points at time zero were measured at the end of the prepulse. The illustration of the protocol in the inset was calculated from the model (see below) with a 100 ms interval at -60 mV between the prepulse and postpulse.
Fig 9

(A) 

-30, -60, -90 mV

Inactivation: ▼ □ △
Recovery: ▼ ■ ▲

Ca^{2+}, short

(B) 

Ca^{2+}, long

(C) 

Ba^{2+}, long

Graphs showing the relationship between b/a and time (ms) for different conditions.
Figure 10. Recovery from inactivation at -30 mV. Symbols represent protocols of different length, and include data also plotted in Fig. 9. The curves drawn correspond to time constants of 400 ms (A) and 1 s (B-C).
Fig 10

A. Ca\textsuperscript{2+}, short

B. Ca\textsuperscript{2+}, long

C. Ba\textsuperscript{2+}, long
Figure 11. Calcium current inactivation calculated from Schemes 1-2. Currents and current-voltage relations are displayed as for Fig. 1 (A) and Fig. 2 (B). The absolute current levels are arbitrary.
TABLE 1

_Recovery from inactivation_

<table>
<thead>
<tr>
<th>Prepulse length (ms)</th>
<th>Ba(^{2+})</th>
<th>Ca(^{2+})</th>
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<td>360</td>
<td>360</td>
<td>75</td>
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<table>
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<tr>
<th>Recovery voltage</th>
<th>Time constant</th>
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<td>(mV)</td>
<td>(ms)</td>
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**Experimental data**

<table>
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<tr>
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<th>Ca(^{2+})</th>
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<tr>
<td>-90</td>
<td>110 ± 20</td>
<td>130 ± 20</td>
<td>110 ± 20</td>
</tr>
<tr>
<td>-60</td>
<td>250 ± 40</td>
<td>230 ± 50</td>
<td>160 ± 20</td>
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<tr>
<td>-30</td>
<td>710 ± 290</td>
<td>710 ± 300</td>
<td>370 ± 60</td>
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</table>

**Model**

<table>
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<tr>
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<th>Ba(^{2+})</th>
<th>Ca(^{2+})</th>
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<tbody>
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<td>-90</td>
<td>116</td>
<td>106</td>
<td>126</td>
</tr>
<tr>
<td>-60</td>
<td>285</td>
<td>204</td>
<td>170</td>
</tr>
<tr>
<td>-30</td>
<td>673</td>
<td>294</td>
<td>*</td>
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</table>

Experimental values are means (± SEM, n=5), from linear regression of the logarithm of the fractional recovery vs. time. Time constants were measured from the protocols of Fig. 8-10, defining zero recovery as the current measured at the end of the prepulse, and full recovery as the point of convergence of the inactivation and recovery protocols. A cutoff of -2 in units was used. Prepulses were given to the voltage generating peak inward current. For model simulations, time constants for recovery were calculated from single exponential fits to the return of channels to the closed state.

*Fit by two exponentials (τ = 72, 401 ms) of equal amplitude.
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