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THE EFFECT OF ISOPROTERENOL ON THE Na-K PUMP ACTIVITY IN ISOLATED VENTRICULAR CARDIAC MYOCYTES IN THE RABBIT

AYMAN Y. FADIL

A Thesis Submitted to the School of Graduate Studies
of the University of Ottawa in Partial Fulfilment
of the Requirements for the Degree of

Master of Science in Physiology

SUPERVISOR:
Dr. M. Désilets

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ISBN 0-315-95917-7

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ABSTRACT

In order to study ß-adrenergic regulation of Na^+ transport in heart, a method was developed to directly measure intracellular free-Na^+ concentration ($[Na^+]_i$) in ventricular myocytes freshly isolated from adult rabbit heart. The approach consisted of injecting the Na^+-sensitive fluorescent indicator SBFI by iontophoresis, and the simultaneous measurement of transmembrane ion current through the use of a single electrode voltage-clamp technique. $[Na^+]_i$ was determined from the ratio of fluorescent intensities measured at 345 and 380 nm. The results demonstrated that the properties of SBFI were markedly altered by the intracellular environment. Thus, the minimum ratio measured in the absence of Na^+ (R_{min}) decreased from 2.56 in the bathing solution, to 1.44 in the cellular milieu. Similarly, the calibration curve shifted to higher $[Na^+]$ values, such that the Na^+ concentration yielding a ratio value corresponding to the mid-point between R_{min} and the ratio measured at saturating Na^+ concentrations (R_{max}), increased from 28.5 to 158 mM.

Using this approach, steady-state $[Na^+]_i$ was found to be 6.4±1.8 mM in myocytes superfused at 37°C with modified Tyrode's solution containing 2 mM Ca^{2+}. Decreasing Ca^{2+} concentration to 0.8 mM caused an increase of $[Na^+]_i$ to 13.0±1.4 mM. The ß-adrenergic agonist isoproterenol (ISO), when applied at a concentration of 0.5 µM, failed to cause any detectable change of steady-state $[Na^+]_i$ or transmembrane current. To test whether this lack of effect also occurred at higher $[Na^+]_i$, cells were exposed to the cation-selective ionophore gramicidin (10 nM), which induced a slow but continuous increase in $[Na^+]_i$. Under those conditions, ISO caused a decrease or even reversed the gramicidin-induced $[Na^+]_i$ rise, while inducing an outward shift of the transmembrane current. On average, the $[Na^+]_i$ rate of change decreased from 4.6±0.9 to -2.7±1.0 mM/min in the presence of 0.5 µM ISO. Similarly, the holding current shifted from -149±88 to 73±86 pA (holding potential kept at -70 mV). These relative changes induced by ISO were not significantly affected by the removal of extracellular Ca^{2+}; -8.0±2.5 mM/min for the ISO-induced changes in $[Na^+]_i$ rate of rise, and 58±17 pA for the transmembrane current. The data also showed a strong correlation between these effects and the $[Na^+]_i$ level at the time of ISO exposure. Under those conditions, strophanthidin (100 µM) completely inhibited the effects of ISO. Washout of intracellular Ca^{2+} by prolonged preincubation of the myocytes in solutions containing 0 mM Ca^{2+}, 0.5 mM EGTA and 25 µM BAPTA-AM caused an important attenuation of the effects of ISO. Thus, the ISO-induced change of $[Na^+]_i$ rate of rise became equal to -2.5±0.6 mM/min, while the holding current did not shift significantly.

This study, therefore, demonstrated the following: 1) ISO directly stimulates the Na-K pump in rabbit ventricular myocytes; 2) the stimulation is likely to occur through an increased turnover rate of the Na-K pump, and; 3) this stimulation is dependent upon intracellular free-Ca^{2+}.

ACKNOWLEDGEMENTS

I would like to thank the many people who helped and supported me, both with this thesis, and many other ways during my stay in Canada. Without them the thesis would not exist, and I would not have made so many valued friends.

Much appreciation is due Dr. Michel Désilets, for his acceptance and guidance, and for the relaxed and creative work environment he maintained. Through many late nights, and much spirited debate, his support was always helpful and enlightening.

Dr. Kenneth Marshall has been a constant source of inspiration and strength throughout my academic life here. I owe much of what I have achieved to him, and will never forget his advice and kindness.

The two members of my Research Advisory Committee, Dr. Mary Morris and Dr. David Parry provided much invaluable support and assistance, and their help was very important to me, particularly when Dr Désilet was on sabbatical.

The project would not have been possible without the invaluable contribution of the "technical experts", Isabella Roy, Diane Chabot, Marise Ramage and Josee Emmerson. Their help and friendship will always mean a lot to me. Thanks also to Phillip Nicholson and Kim Woodill for their help with the design, layout and production of this document.

Many friends and colleagues have provided me with encouragement, assistance and support. Douglas and Judy Eley, Reena Sandhu and David and Betty Van Dorp provided the spark to get me started, and the emotional fuel to keep me going. Diane and Douglas Maulden, Kim Nicholson and Brent Hodgkinson also encouraged and supported me throughout. Mya Lang, Andrea McKay and Allison McKay were very helpful and provided me with much needed encouragement during some very trying times — fortunately they didn't ask me to pay their long distance phone bills.

A special word from the heart for my parents — thank you for everything.

I am very grateful to Mustafa Hariri and Dr. Abdul-Rahman Bakhsh for their continual assistance and moral support, particularly in times of stress and personal difficulties.

Special acknowledgement to the Medical Research Council of Canada and Dr. Bakhsh Hospital of Jeddah, Saudi Arabia, for providing the funding for this research project.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AM	acetoxymethyl ester
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BAPTA	*bis*-(*o*-aminophenoxy)-ethane-N N N'N'-tetraacetic acid
BTX	batrachotoxin
C	coulomb
$[Ca^{2+}]$	calcium ion concentration
$[Ca^{2+}]_i$	intracellular calcium ion concentration
$[Ca^{2+}]_o$	extracellular calcium ion concentration
cm	centimeter
EDTA	ethylenediaminitetraacetic acid
EGTA	ethylene glycol bis(ß-aminoethylether)N N N'N'-tetraacetic acid
Gram.	gramicidin
Hz	Hertz
I_m	transmembrane current
ISO	isoproterenol
$[K^+]$	potassium ion concentration
$[K^+]_i$	intracellular potassium ion concentration
$[K^+]_o$	extracellular potassium ion concentration
k_{app}	apparent dissociation constant

LIST OF ABBREVIATIONS (continued)

k_d	dissociation constant
kg	kilogram
kHz	kilohertz
M	molar
min	minute
ml	millilitre
μM	micromolar
mM	millimolar
MΩ	megaohm
nA	nanoampere
$[Na^+]$	sodium ion concentration
$[Na^+]_i$	intracellular sodium ion concentration
$[Na^+]_o$	extracellular sodium ion concentration
Na-K pump	sodium-potassium pump
nm	nanometer
NMR	nuclear magnetic resonance
pA	picoampere
Pi	inorganic phosphate
R	ratio of fluorescence
R_{100}	ratio at 100mM
R_∞	ratio at saturating concentrations of Na^+
R_{max}	maximum ratio

LIST OF ABBREVIATIONS (continued)

R_{min}	minimum ratio
SBFI	sodium-binding benzofuran isophthalate
sec	second
SEM	standard error of the mean
STR	strophanthidin
V_m	membrane potential
V_{max}	maximum turnover rate

INTRODUCTION

ROLE OF Na^+ IONS IN HEART

Na^+ ions play a central role in the generation and propagation of electrical activity in heart. It is well known that in ventricular and atrial myocytes, the depolarisation phase of the action potential is caused by an influx of Na^+ ions following the activation of the voltage-sensitive Na^+-channels (Fozzard and Hanck, 1991).

Maintenance of the Na^+ concentration gradient is also essential for the regulation of several important intracellular ion species, such as Ca^{2+}, H^+, Cl^- and Mg^{2+} (Vormann and Gunther, 1987; Murphy et al., 1991) and inorganic phosphate (Medina and Illingworth, 1980; Ponce-Hornos and Langer, 1982).

Control of intracellular Ca^{2+} by the Na^+-Ca^{2+} exchanger is now well recognized, and is highly sensitive to the Na^+ electrochemical gradient (e.g. Sheu and Fozzard, 1982). This transport mechanism is electrogenic by exchanging 3 Na^+ ions per Ca^{2+} ion (Eisner and Lederer, 1985). Under normal conditions it promotes Ca^{2+} efflux in exchange for entry of Na^+ ions. During the plateau phase of the action potential (Shattock and Bers,1989; Bers, 1991; Wier, 1991), the exchanger can reverse and cause the net entry of Ca^{2+} ions. It should be mentioned that the activity of this exchanger alone can contribute significantly to transmembrane Na^+ movement. Thus, Deitmer and Ellis (1978b) demonstrated that

raising external Ca^{2+} concentration not only caused a decrease of steady-state $[Na^+]_i$ in Purkinje fibres, but also could prevent the increase of $[Na^+]_i$ induced by Na-K pump inhibition.

It is now also well accepted that the Na-H countertransport mechanism is a major pH regulator in heart muscle (Ellis and MacLeod, 1985; Piwnica-Worms et al., 1985). This exchanger is amiloride-sensitive (Ellis and MacLeod, 1985; Kaila and Vaughan-Jones, 1987). Due to its high dependence on intracellular pH, the exchanger is largely inhibited at normal intracellular pH, but becomes a powerful proton extrusion mechanism upon slight acidification (Wallert and Frohlich, 1989; Vaughan-Jones and Wu, 1990). Under those conditions, the exchanger also becomes an important pathway for Na^+ influx. In fact, the consequent increase of Na^+ influx and, hence, of $[Na^+]_i$ has been implicated as a major mechanism for Ca^{2+} overload induced by intracellular acidosis (Dennis S.C. et al., 1990; Vaughan-Jones and Wu, 1989). The fact that the Na^+ movement through this exchanger may be significant can be inferred from experiments showing that intracellular acidification is followed by an increase of $[Na^+]_i$ that can be blocked to a large extent by amiloride inhibition of the exchanger (Kaila and Vaughan-Jones, 1985b, 1987; Bielen et al., 1990). Na^+ also contributes to the regulation of intracellular pH through the recently suggested Na-HCO_3 symport mechanism (Lagadic-Gossmann et al., 1991, 1992a; Dart and Vaughan-Jones, 1992).

The presence of some Na^+-dependent Cl^- transport mechanisms in the heart is also probable, although few studies have been performed as yet. In cultured heart cells,

transmembrane Cl^- flux is largely electrosilent (Piwnica-Worms et al., 1983), and is carried mainly by the furosemide-sensitive Na-K-Cl cotransport (Liu et al., 1987) and the stilbene-sensitive Na-dependent Cl-HCO_3 exchange (Liu et al., 1990). Measurements in papillary muscles showed that Cl^- is not passively distributed (e.g. Baumgarten and Fozzard, 1981; Caillé et al., 1981; Fong and Hinke, 1981), and this excess Cl^- content has indeed been shown to be reduced by Na-Cl cotransport blockers (Baumgarten and Duncan, 1987). In any case, the presence of these various Na^+-dependent Cl^- transport mechanisms demonstrates the importance of Cl^- regulation in heart which, in turn, is likely related to the control of intracellular pH and cell volume (Drewnowska and Baumgarten, 1991; Clemo et al., 1992), as observed in other tissues (Brodsky, 1980).

In summary, it is clear that heart function is highly dependent upon the close regulation of the Na^+ electrochemical gradient. This is important not only for the electrical activity of the heart, but also for its contractile activity via the control of intracellular Ca^{2+}, and for its metabolic and cellular functions through regulation of intracellular pH and cell volume. In addition, it should be mentioned that intracellular Na^+ ions may also directly act as modulators of enzyme function. Thus, Harvey et al. (1991), recently demonstrated an important effect of intracellular Na^+ on the activation by ß-agonists of cAMP-dependent channels.

Since sodium plays such an essential role in regulating intracellular functions, it is important to make a detailed study of the mechanisms that regulate the intracellular concentration of sodium in the cardiac myocytes.

THE Na-K PUMP

The influx of Na^+ during the electrical activity of cardiac cells, together with the activity of various Na^+-dependent transmembrane carriers, implies the presence of a continuous inward Na^+ leak, which must be counterbalanced in order to maintain the Na^+ concentration gradient. The balance between this leak and the Na^+ extrusion is what determines the sodium regulation in myocardium. Under normal conditions, this active extrusion mechanism is carried out by a unique but powerful ion exchanger; the Na-K pump.

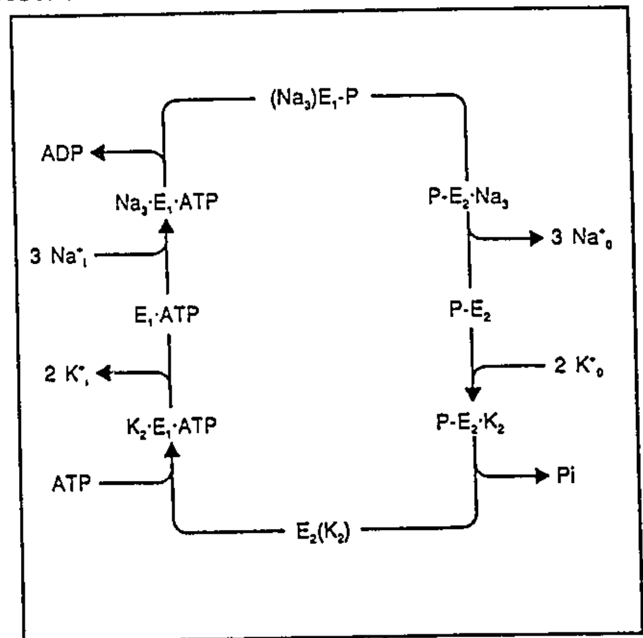
A. Structure:

It is well known that Na^+ regulation in heart cells is performed through the Na-K ATPase that allows active Na^+ extrusion in exchange for K^+ ions. The structure of this ubiquitous membrane protein consists of two subunits, α- and β-, arranged in a one-to-one stoichiometry (Jorgensen, 1982). The α subunit is responsible for the hydrolysis of ATP and the translocation of Na^+ and K^+ ions. The glycosylated β-subunit is not required for the activity of the pump, and its precise role remains to be defined, although it has been postulated that it may be involved in the synthesis or assembly of the α-molecules (Noguchi et al., 1990; but see Ackermann and Geering, 1992). Recently, Lutsenko and Kaplan (1992) also provided evidence that the β-subunit has a modulatory role on the

activity of the pump. Both subunits have at least 3 distinct isoforms the expression of which varies extensively between cells and tissues (see Sweadner, 1989; Lingrel at al., 1990). For instance, the α-subunit in adult rat heart tissue is represented only by the α1 and α2 isoforms, while the fetal and neonatal hearts also express the α3 isoform (Orlowski and Lingrel, 1988).

B. <u>The Post-Albers model</u>:

The biochemical properties of the Na-K ATPase as a transmembrane ion carrier have been studied extensively. It is now well accepted that the net enzymatic reaction leads to the hydrolysis of one molecule of ATP, and results in the extrusion of three Na^+ ions in exchange for the entry of two K^+ ions. The basic intermediary steps involved in the reaction are illustrated in the scheme presented below.



This scheme is a simplified version of the Post-Albers model (Albers, 1967; Albers et al. 1968; Post et al., 1969) which has now received extensive experimental support (see Stein, 1986; Glynn, 1988; Sachs, 1991). Basically, the enzyme can exist into two conformational states, the E_1 and E_2 forms, which can bind intracellular

Na^+ (Na^+_i) and extracellular K^+ (K^+_i), respectively. The binding of 3 Na^+ ions to the E_1·ATP complex leads to the phosphorylation of the protein, and the release of ADP, together with the occlusion of the Na^+ ions, the $(Na_3)E_1$-P complex. This state of occlusion, whereby the ions are not accessible for exchange, is unstable, and spontaneously transforms into the E_2 configuration, releasing the Na^+ ions to the extracellular milieu. The dephosphorylation of the E_2 form, i.e. the release of inorganic phosphate Pi, occurs once K^+ ions bind to the complex. This, in turn, causes the occlusion of the K^+ ions, E_2 and K_2, and the subsequent transformation of the E_2 state into E_1. ATP, more specifically MgATP, binding accelerates this transformation and the deocclusion and dissociation of K^+ ions on the intracellular side.

C. Affinity of the pump to ligands:

The series of reactions schematically representing the pump cycle readily imply that there are 5 important ligands controlling the activity of this pump: Na^+, K^+, ATP, ADP and Pi. Given the complexity of the reactions, the apparent affinity of these ligands can vary extensively, according to the experimental conditions used for their measurements. For instance, and as reviewed by Stein (1986), biochemical measurements of the apparent affinity constant for Na^+ led to values that varied from 0.5 to 20 mM. Recent determinations in intact cardiac myocytes from either the Na^+ flux or current measurements, led to a range of values from 8 to 43 mM (Sejersted et al, 1988; Nakao

and Gadsby, 1989; Mogul et al., 1990). As noted by Mogul et al. (1990), this apparent discrepancy may be explained by the various levels of intracellular K^+ present when the pump activity was measured, owing to the competitive effect of K^+ ions on intracellular binding sites of the pump for Na^+ ions. In any case, these measurements indicate that intracellular Na^+ can be an important regulator of the pump activity, since the physiological concentration of Na^+ in cardiac myocytes is about 8 to 15 mM (e.g. Cohen et al., 1982; Sheu and Fozzard, 1982; Désilets and Baumgarten, 1986a,b; Shattock and Bers, 1989), a value likely far below that required for saturation of the pump. This implies that small variations in $[Na^+]_i$ can substantially affect the rate of the pump cycle (see also Stimers et al., 1990a).

On the other hand, the extracellular K^+ concentration yielding half-maximal activation of the pump in heart is about 1 mM (e.g. Gadsby, 1980; Eisner et al., 1981b; Glitsch et al., 1982; Falk and Cohen, 1984); a value 4 to 5 times lower than the physiological concentration of extracellular K^+(see Eisner and Smith, 1991; Katz, 1992). This implies that increasing extracellular K^+ levels should have a relatively small stimulatory effect on the Na-K pump activity.

As far as we know, the apparent affinity constant for ATP has not been determined in intact tissues, but biochemical studies on membrane preparations gave a value range of approximately 1 to 20 μM (Robinson, 1976; Karlish et al., 1978). Since the normal intracellular concentration of ATP is about 5 mM (Katz, 1992), this indicates that, with all other parameters remaining constant, even drastic changes of intracellular ATP should

have little effect on the pump cycle rate. On the other hand, the apparent affinity for intracellular Pi has been reported to be about 20 mM (Robinson et al., 1978). Intracellular concentration of Pi is about 4 mM in heart (Polgreen et al., 1993) so that its increase should have an inhibitory effect on pump activity (Garay and Garrahan, 1975; Ponce-Hornos and Langer, 1982). Little information regarding the affinity of ADP for the pump is available, although a value of about 10 μM, measured in the absence of ATP and at 0°C, has been reported (Taniguchi and Post, 1975). It should be noted, however, that the intracellular concentration of ADP, estimated to be in the order of 0.02 mM (Katz, 1992), remains relatively constant in the cell, due to the strong intracellular ADP buffering system (see Chance et al., 1986; Mowbray and Patel, 1991).

In summary, the pump rate cycle, as for any chemical reaction, depends upon the concentrations of its ligands. Among those, intracellular Na^+ probably constitutes the most important modulator of pump activity, owing to the fact that its physiological concentration approximates its affinity constant. Furthermore, decreasing the extracellular K^+ concentration or increasing intracellular Pi levels should have inhibitory effects on the pump activity.

D. Electrogenicity of the pump.

The fact that the reaction cycle of the Na-K ATPase leads to the exchange of 3 Na^+ for 2 K^+ implies the net extrusion of one positive charge per cycle and, hence, gives rise

to another important property of this transporter; its electrogenicity. It is now well recognized that the pump can act as a transmembrane current generator. This pump current, which has been measured in several tissues (see Thomas, 1972; De Weer, 1984) including heart (e.g. Gadsby et al., 1985; Gadsby and Nakao, 1989; Nakao and Gadsby, 1989; Mogul et al., 1990; Stimers et al., 1990b; Gao et al., 1992), can substantially affect the membrane potential (see Glitsch, 1982; Gadsby, 1984). Conversely, the electrogenic nature of the Na-K pump makes it sensitive to membrane potential (Rakowski and Paxson, 1988; Gadsby and Nakao, 1989). Thus, Gadsby and Nakao (1989) reported that a depolarisation of cardiac myocytes causes an increase of the pump activity, with a maximal stimulation occurring at about 0 mV.

E. Modulation of pump activity by intracellular Ca^{2+} and pH:

It has long been recognised that Ca^{2+} ions can inhibit isolated Na-K ATPase (Skou, 1957). The Ca^{2+} concentrations needed to inhibit the enzyme usually varied from 0.1 to 1 mM (Huang and Askari, 1982) however, so the physiological significance of this inhibition was difficult to explain. If this inhibitory effect occurred at a site accessible to extracellular Ca^{2+}, then the enzyme would be largely inhibited in most of the preparations. Conversely, an intracellular site with such low affinity would preclude any physiological role for Ca^{2+} ions. Some evidence that the activity of the Na-K ATPase in intact membranes can be inhibited by physiological levels of intracellular free-Ca^{2+} does exist.

The most direct indications come from the work of Yingst and collaborators on human red blood cells (Yingst and Polasek, 1985). They showed that concentrations as low as 0.2 μM could have a significant inhibitory effect on the Na-K ATPase activity. Inhibition of the pump by physiological concentrations of Ca^{2+} has also been demonstrated in rat hepatocytes (Berthon et al., 1985) and myometrium (Turi and Somogyi, 1988). Yingst and Polasek (1985) demonstrated that erythrocytes Na-K ATPase sensitivity to low concentrations of Ca^{2+} was substantially increased by the presence of an extrinsic protein, thereby concluding that Ca^{2+} ions could be a physiological regulator of Na-K pump, through the intermediary of a protein factor. Recent studies by Yingst and peers (Yingst et al., 1992) determined that this protein was most likely calmodulin. This putative effect of calmodulin remains to be demonstrated in other tissues, although homogenates of various preparations, including heart, enhanced Ca^{2+}-induced inhibition of the Na-K ATPase from human red blood cells (Yingst, 1988). It is also interesting to note that this Ca^{2+} sensitivity may be specific to the α2-isoform of the Na-K ATPase (Turi et al., 1991) and, hence, tissue and species-dependent.

The effect of intracellular pH on Na-K pump activity in heart is another unresolved issue. Although the results are variable, biochemical studies of Na-K ATPase generally demonstrate a pH sensitivity of its activity (Skou, 1957; Dunham and Glynn, 1961), the optimal range generally occurring at the physiological pH. Studies on squid giant axon (Breitweiser et al.,1987) corroborated these results by showing an important inhibition of ouabain-sensitive K^+ and Na^+ fluxes when intracellular (but not extracellular) pH was

varied from the optimal value of pH 7.2-7.4. However, Godfraind et al. (1977) found that Na-K ATPase isolated from guinea-pig heart was not significantly affected by pH variances from 6.4 to 7.4, unless the Ca^{2+} concentration was higher than 10^{-6} M. This would imply a limited effect of pH (and Ca^{2+}) under normal conditions. It remains to be determined if this insensibility to pH also occurs in intact cardiac preparations. In fact, Bielen et al. (1990) indicated that intracellular acidification of rabbit cardiac Purkinje cells suppressed the rate of active Na^+ extrusion and Na-K pump current, suggesting a direct inhibitory effect of intracellular pH on Na-K pump activity.

REGULATION OF INTRACELLULAR Na^+ BY CATECHOLAMINES.

Through their important inotropic and chronotropic effects on the heart, catecholamines have proven to be the most potent regulator of heart function. They affect their target organs mainly through α and β adrenergic receptors. It is generally accepted that β-adrenergic activation is far more important than that of α in regulating myocardial functions (Katz, 1992). While β-blockers inhibit catecholamine stimulation of the heart function, α-blockers leave it intact (Feigl, 1989). However, α-adrenergic modulation has a weak positive inotropic effect (Katz, 1992), and has been implicated in affecting cardiac rhythm (Rosen et al., 1991).

The mechanisms involved at the cellular level of the β-modulation are complex and numerous. Among the best known effects are; the increase of metabolic activity through glycolysis (Clark and Patten, 1984) and glycogenolysis (Williamson, 1964; Katz, 1983), enhancement of the transient Ca^{2+} current (Reuter and Scholz, 1977; Tsien et al. 1986; Hartzell and Duchatelle-Gourdon, 1992) concurrent with stimulation of Ca^{2+} uptake in intracellular stores, and with decreased myofilaments sensitivity to Ca^{2+} (Fabiato and Fabiato, 1979). These effects result in an increase of contractile force and an acceleration of the rates of both contraction and relaxation, thereby enhancing the effectiveness of the heart as a pump. Also important is the ß-adrenergic stimulation of the pacemaker current present in nodal cells (Brown and DiFrancesco, 1980), Purkinje fibres (Chang et al., 1991) or even atrial (Earm et al., 1983) and ventricular myocytes (Yu et al., 1993). Several K^+

currents have also been shown to be regulated by ß-agonists, including; the delayed rectifier (Kameyama et al., 1985), the transient outward current (Nakayama et al., 1989) or a background K^+ conductance (Boyden et al., 1983; Gadsby, 1983). Finally, one should mention the recent and important discovery of a Cl^- current activated by ß-agonists (Bahinski at al., 1989; Harvey et al., 1990). All these effects occur, at least partially, through the well known increase of cAMP levels by ß-adrenergic agonists (Lefkowitz et al., 1983) and the consequent activation of protein kinase A (see Shabb and Corbin, 1991).

There is also increasing evidence that Na^+ homeostasis may be substantially affected by catecholamines. Given the central role played by Na^+ in both electrical and contractile activities, such a modulation of transmembrane Na^+ movement could be of important physiological significance in maintaining proper heart function during adrenergic activation. The following subsections describe specific transmembrane Na^+ pathways that are believed to be under the direct influence of catecholamines.

A. Effects on the Na-K pump:

It is generally accepted that catecholamines stimulate the active Na^+ efflux in skeletal and cardiac muscle (see 16, Gick et al., 1988). In heart preparations, catecholamines have been shown to decrease $[Na]_i$ (Wasserstrom et al., 1982; Pecker et al., 1986). This effect presumably occurs through β-receptors, since it is inhibited by propranolol (Wasserstrom et al., 1982), and can be mimicked by cAMP analogues (Pecker

et al., 1986). It should be mentioned, however, that α-adrenergic stimulation of the Na-K pump has also been postulated (Shah et al., 1988).

The mechanisms accounting for this stimulation are still controversial, and will require much more investigation before they are widely accepted. It has been suggested that the stimulation is indirect, and occurs through an increase of K^+ conductance that would lead to an accumulation of extracellular K^+ (Glitsch et al.,1989). Conversely, a direct stimulation of active efflux has been postulated, since the catecholamine-induced decrease of intracellular Na^+ activity occurs even at high concentrations of external K^+, where accumulation of K^+ would have little effect on Na-K pump activity (Pecker et al., 1986). In agreement with these results, Désilets and Baumgarten (1986b) found an important isoproterenol-induced decrease of intracellular Na^+ activity in isolated ventricular myocytes. Furthermore, when Barrette et al. (1990) measured the K^+-induced pump current, they found a significant acceleration of the relaxation of this transient outward current. It was also found that this effect was negated by the removal of external Ca^{2+}, thereby indicating that the β-agonist-induced stimulation of the Na-K pump occurs through a Ca^{2+}-dependent mechanism. These experiments have recently been confirmed by Gao et al. (1992), who demonstrated a dual effect of the ß-agonist isoproterenol on the Na-K pump current, depending upon the level of intracellular Ca^{2+}. This dependency on intracellular Ca^{2+} could explain the lack of effect of isoproterenol on Na-K pump current reported by Bahinski and Gadsby (1990), since these authors strongly buffered intracellular Ca^{2+} with EGTA in their dialysing pipettes. As the involvement of Ca^{2+} in the

β-adrenergic effects on active extrusion of Na^+ from the cell has not yet been fully determined, an important objective of the current research project is to test the hypothesis that Ca^{2+} is required for isoproterenol-induced stimulation of the Na-K pump.

B. Effects on Na^+ influx:

There are several Na^+ influx pathways which can be regulated by catecholamines. Thus, the TTX-sensitive Na^+ current is inhibited by β-agonists through a left shift of its steady-state inactivation curve (Schubert et al., 1989; Ono et al., 1993). This implies that intracellular Na^+ concentration should decrease under adrenergic conditions, and could contribute to the observed effects of catecholamines on intracellular Na^+ activity.

Although cAMP-dependent Na^+ conductances have been shown in several types of tissues (e.g. Connor and Hockberger, 1984; McCrohan and Gillette, 1988), the existence of such a conductance in ventricular muscle is not yet firmly established. Egan et al (1988) demonstrated an important cAMP-dependent background conductance that was inhibited by the removal of external Na^+. However, it is quite likely that this conductance is in fact carried by Cl^- ions, as observed in guinea-pig and rabbit myocytes (Harvey and Hume, 1989; Matsuoka et al., 1990).

Several Na^+ dependent transport mechanisms could also be under adrenergic influence. The Na-H exchanger is a likely candidate, as it is influenced by a variety of hormonal stimuli in other preparations (see Sergeant et al., 1989). In red blood cells,

cAMP can either elicit a stimulation (Boyett et al., 1986) or an inhibition (Sergeant et al.,1989) of the exchanger. In guinea-pig ventricular myocytes, adrenaline stimulated the Na-H antiporter (Lagadic-Gossmann et al., 1992b). This effect was probably carried out through an α-pathway, since it could be mimicked by the α-agonist phenylephrine, and inhibited by the α_1-antagonist prazosin (Lagadic-Gossmann and Vaughan-Jones, 1993). Conversely, under the same conditions, specific β-activation through the β-agonist isoproterenol inhibited the exchanger (Lagadic-Gossmann and Vaughan-Jones, 1993). Partial inhibition of the Na-H exchanger by β-adrenergic agonists was also observed in sheep Purkinje fibre (Guo et al., 1992). Adrenaline inhibited the Na-HCO_3 cotransporting mechanism in ventricular myocytes (Lagadic-Gossmann et al., 1992b). This inhibition was mimicked by the α-agonist phenylephrine, and prevented by the α_1-antagonist prazosin (Lagadic-Gossmann and Vaughan-Jones, 1993). However, the β-agonist isoproterenol stimulated the Na-HCO_3 symport, suggesting a β-adrenergic activation pathway for this electrosilent cotransporter (Lagadic-Gossmann and Vaughan-Jones, 1993).

Catecholamines have been shown to be a regulator of Na-Cl transport mechanisms in other cell types (e.g. Boyett et al., 1986), and these potentially important effects need to be evaluated in heart muscle.

All these Na^+ influx mechanisms should be taken into consideration when studying the effects of β-activation on $[Na^+]_i$. As the major focus of this research project is to study the β-activation of the Na-K pump, the information obtained should be interpreted carefully, to ensure that changes due to these other Na^+ influx pathways are not included.

C. Isoproterenol

Isoproterenol is a synthetic sympathomimetic drug which mimics the effects of catecholamine on β-adrenergic receptors (Hoffman, 1987), and has positive chronotropic and inotropic effects on the myocardium (Katz, 1992). Like other β-agonists, isoproterenol activates the β-receptor, which leads to stimulation of the adenylate cyclase via a stimulatory guanine nucleotide binding protein (G_s). This, in turn, increases the conversion of ATP to cAMP (Susanni et al., 1991). An increase in intracellular cAMP activates the protein kinase A, which in turn modulates several cellular functions, including transmembrane transporting mechanisms, by modulating cellular enzymes and ion channels (Katz, 1992).

As noted previously, the mechanisms accounting for the stimulation of the Na-K pump by the β-agonists are yet to be clarified. Since studying the effects of β-adrenergic stimulation on the Na^+-K pump is a major focus of this project, the use of isoproterenol to clarify some of these mechanisms seems appropriate, due to its specificity to β-receptors (Feigl, 1989).

PHYSIOLOGICAL MEASUREMENTS OF THE Na-K PUMP ACTIVITY.

As described below, three basic approaches have been employed for studying the activity of the Na-K pump in intact tissues; radioisotope determinations of the ouabain-sensitive K^+ and Na^+ fluxes, measurements of intracellular Na^+ activity and electrical recordings of the transmembrane current generated by the Na-K pump. In addition, two of the recent techniques which have been developed for the measurement of the Na^+ content in myocardial tissue should be noted. One, nuclear magnetic resonance (NMR), has been used to monitor ^{23}Na in isolated perfused hearts (Kohler et al., 1991), while the other, X-ray microanalysis, has also been applied to determine the $[Na^+]_i$ in various heart preparations (Warley, 1991; LeFurgey et al., 1992). Although both techniques can provide important information regarding Na^+ homeostasis in the myocardium, they have yet to be used for the study of the transmembrane movement of Na^+. This is probably related to the complexity of these sophisticated techniques, as well as the fact that they either measure Na^+ only in whole tissues, as with the NMR technique, or they necessitate the destruction of the cell preparations as is the case with X-ray microanalysis.

A. Tracer flux measurements:

The first approach, consisting of tracer flux measurements, has been extensively used in several preparations (e.g. Brinley and Mullins, 1974; Rakowski, 1989) but not in

heart, probably due to the complex structure of the organ, together with its instability during inhibition of the Na-K pump. Nevertheless, it is interesting to note that as early as 1965, Glitsch et al. (1965) demonstrated through this method that ouabain-sensitive K^+ and Na^+ flux rates were enhanced by adrenaline in Purkinje fibres. This result was also confirmed later in other heart preparations using a similar approach (Vassalle and Barnabei, 1971; Hougen et al., 1981). There are important limitations related to this type of study in heart, however. First, one has to assume homogeneity of the cell population. Second, radioisotope measurements reflect an overall distribution of the tracers in the exchangeable compartments, such that the flux rates may be a composite of fluxes from these various compartments (which may include extracellular binding, trans-sarcolemmal exchange, intracellular binding and uptake in organelles, etc.). Third, an estimate of the Na-K pump activity with this approach implies the use of an inhibitor of the pump and, hence, relies on the hypothetical specificity of this inhibitor. More importantly, an inhibition of the pump causes a change of the membrane potential, and a consequent indirect change of K^+ and Na^+ transmembrane movements. Further, tracer flux studies must be performed under steady-state conditions in order to lead to unequivocal conclusions (see Borle, 1981), a condition which cannot be achieved during pump inhibition, since it would cause a progressive dissipation of the ionic gradients.

B. Ion-selective microelectrodes and voltage-clamp in multicellular preparations:

The second approach, direct measurement of cytosolic Na^+ concentration, obviously circumvents several of the limitations related to radioisotope measurements. This has been achieved following the development of ion-selective microelectrodes. This method, which is still used to study Na^+ homeostasis in multi-cellular preparations (e.g. Wilde and Kleber, 1991), offers the advantage of directly measuring the intracellular free-Na^+ concentration. This is technically difficult because it requires stable dual impalements with both an ion-selective microelectrode and a conventional one, to record the transmembrane voltage (see Dagostino and Lee, 1982). Nevertheless, the use of this technique must be considered as a major breakthrough for our present understanding of intracellular Na^+ regulation in heart. Thus, through the pioneering work of Eisner, Lederer and collaborators in cardiac Purkinje fibres (Eisner and Lederer, 1980; Eisner, et al. 1981a), not only was the active extrusion of Na^+ through the Na-K pump characterized (Eisner and Lederer, 1980; Glitsch et al., 1981; Sejersted et al., 1988), but also the contribution of several Na^+ influx pathways, including the voltage-gated Na^+ channels during electrical activity (Cohen et al., 1982), and the Na-Ca (Sheu and Fozzard, 1982, Shattock and Bers, 1989), the Na-H (Kaila and Vaughan-Jones, 1987), and the Na-Mg (Murphy et al., 1991) exchange mechanisms, were evaluated. The studies of Eisner et al. (1981a) and Glitsch et al., (1981) were also important because they were done under voltage-clamp conditions, thereby allowing the simultaneous recording of transmembrane ion current. The electrical current generated by

the pump could thus be clearly defined and studied, an approach also used by Gadsby and co-workers (Gadsby and Cranefield, 1979; Gadsby, 1980).

Besides the technical difficulties, which required highly skilled experimenters, these delicate studies also had other limitations. First, voltage-clamp experiments were essentially limited to the Purkinje fibres (and a few on amphibian atria: Akasu et al., 1978), the only easily available heart preparations that are small enough to be properly voltage-clamped. Thus, this approach has never been used to study the regulation of the Na-K pump in ventricular myocardium. Secondly, ion-selective microelectrodes record Na^+ concentrations in only a very localized region surrounding the tip, and the reliability of the measurements obtained with this technique is based on the assumption of the absence of intracellular concentration gradients or cellular heterogenicity within these multicellular preparations. Finally, and most importantly, the small intercellular clefts present in multicellular cardiac preparations (Sommer and Jennings, 1991) preclude the possibility of close control over the ionic composition of their extracellular environment.

The latter problem is particularly relevant to studies of the Na-K pump, where its inhibition or activation can cause substantial accumulation or depletion of K^+ ions in the extracellular space (Rasmussen et al., 1986), and affect both membrane conductance and pump activity. This potential accumulation-depletion problem is illustrated by several unresolved controversies, which highlight the limitations of the technique. One of these pertains to the aforementioned stimulation of the Na-K pump activity by catecholamines, and may suggest that it could be due, at least partially, to an extracellular accumulation

of K^+ following a catecholamine-induced increase of K^+ conductance. A related example stems from the results of Akasu et al. (1977; 1978) which show that the ß-adrenergic stimulation of the Na-K pump appears to occur through an increase of K^+ affinity at the Na-K ATPase binding sites. This conclusion is important because it points directly to what the author suggested as a possible mechanism involved in the interaction between catecholamines and the Na-K pump. However, it is conceivable that this apparent increased affinity for external K^+ may simply be due to, or at least affected by, concomitant accumulation of K^+ in the intercellular spaces during the measurements of pump current as a function of the concentration of K^+ in the superfusion chamber. This was in fact substantiated by a later report from the same group (Hasuo et al., 1988) which clearly demonstrated similar indirect effects of acetylcholine in their atrial preparation. Another controversy arising from the accumulation-depletion problem is directly related to the measurements of the apparent affinity of the pump for extracellular K^+. As noted previously, the K^+ concentration yielding half-maximal activation is believed to be about 1 mM. This value is consistent with the measurements made on steady-state Na^+ concentration in Purkinje fibres (Deitmer and Ellis, 1978a; Eisner et al., 1981b; Glitsch et al., 1981), but not on transient changes of Na^+ and pump current, where a value of about 5 mM was found (Eisner and Lederer, 1980; Eisner et al., 1981a). This discrepancy could be explained by the depletion of K^+ ions during pump activation, so that the second approach would overestimate the apparent affinity constant. However, Cohen et al. (1987) demonstrated theoretically that this discrepancy could be explained by an effect of

intracellular Na^+ on pump activity, and that large systemic errors in estimates of K^+ affinity can occur and vary according to the experimental approach used.

C. Single cells and patch-clamp:

Clearly, a precise understanding of Na-K pump regulation could only be obtained through conditions where the problem of accumulation-depletion could be abolished. Furthermore, as it is important to control the membrane potential when studying trans-membrane ion movements, these experiments on multicellular cardiac preparations were limited to only a few specialized tissues. The difficulties were minimized, however, by using enzymatically dissociated cardiac myocytes, a technique originally developed by Powell and collaborators (Gould and Powell, 1972). In fact, the possibility of studying viable single cells, together with the development of patch-clamp technique (Hamill et al., 1981), led to a revolution in the field of cardiac electrophysiology, as it allowed, for the first time, close control of membrane potential and, hence, a direct measurement of transmembrane ion currents. Using this approach, electrophysiological characterizations of the major cardiac tissues have been performed. These include studies on ventricular (e.g. Hescheler and Trautwein, 1989) and atrial (Belardinelli et al., 1989) myocytes, pacemaker cells (Irisawa, 1987), as well as cells from Purkinje fibres (Fozzard et al., 1987). Not surprisingly, the current generated by the Na-K pump was also scrutinized, with the most important studies coming from the work of Gadsby and collaborators

(Gadsby et al., 1985; Nakao and Gadsby, 1986; Bahinski and Gadsby, 1988; Gadsby and Nakao, 1989; Bahinski et al., 1990). Their method consisted of measuring this current under voltage-clamp conditions where the membrane ion conductances were maximally inhibited with various channel blockers, and the intracellular ion content was controlled by intracellular perfusion with appropriate solutions contained in large suction pipettes. Thus by "clamping" intracellular Na^+ concentration, this technique allowed them to characterize the dependency of Na-K pump current on membrane potential (Gadsby et al., 1985) and intracellular Na^+ concentration (Gadsby and Nakao, 1985). The technique also provided new insight regarding the voltage-dependent steps involved in the translocation of Na^+ by the Na-K ATPase (Nakao and Gadsby, 1986).

Despite the important information coming from these studies on active Na^+ transport, such experiments are not without difficulties and limitations. First, the method measures only the current inhibited by cardiac glycosides, and is therefore dependent upon the specificity of these inhibitors. Secondly, the pump current is relatively small compared to the other membrane currents, which means that the blockers of these various conductances must also be present during the measurements (e.g. Ba^{2+}, Cs^+ and tetraethylammonium to block K^+ channels, Cd^{2+} to block Ca^{2+} channels, TTX to block Na^+ channels, replacement of Cl^- with large inorganic anions, etc.; see Gadsby et al., 1985). It is still unknown whether these agents could affect the pump properties. Thirdly, intracellular perfusion (which bypasses the need to simultaneously measure Na^+ levels) also implies the loss of essential cell components, including intracellular messengers that

can potentially affect Na^+ regulation. Furthermore, it is also quite likely that concentration gradients between the pipette and the cytosol arise during pump activation, which would produce some uncertainties in experiments such as those involving the determination of intracellular Na^+ affinity (see Nakao and Gadsby, 1989). Finally, this electrophysiological approach can only investigate rheogenic ion transports, i.e. such an approach could not be used to study electroneutral transport mechanisms such as the Na-H exchanger and Na^+-dependent Cl^- transports.

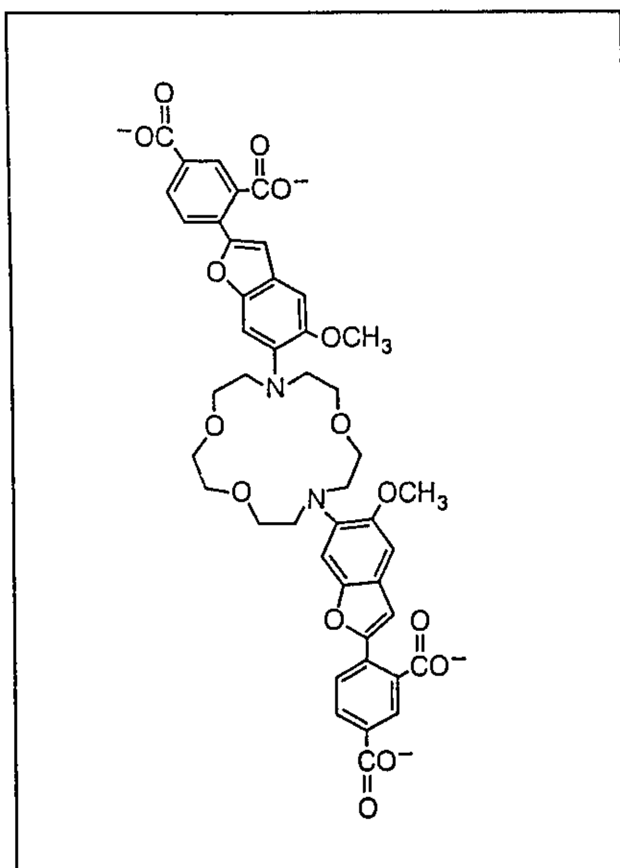
D. Fluorescence determinations of intracellular Na^+:

As noted above, the possibility of simultaneously recording transmembrane currents and cytosolic Na^+ concentration in single cells would offer several advantages for the study and characterization of the Na^+ regulation in cardiac myocytes. Attempts to measure intracellular Na^+ activity in single cells with ion-selective microelectrodes have been successful (Désilets and Baumgarten, 1986a; 1986b) but this method proved to be difficult because of the small size of the preparation.

A promising alternative approach also emerged during that period; the ion-sensitive fluorescent indicators. These indicators, initially developed for the measurement of pH (Rink et al., 1982) and free-Ca^{2+} (Grynkiewicz et al., 1985), could be loaded intracellularly, either directly from a suction pipette, or through the use of membrane permeant forms that could be cleaved by intracellular enzymes and consequently trapped

inside the cells. These latter forms came from the ingenious design of indicators with polar groups that make them water-soluble and membrane-impermeant, but which can be masked by nonpolar protecting groups hydrolysable by the cytoplasm, thereby allowing cell loading by simple incubation. The protecting groups that proved to be particularly useful are acetoxymethyl esters (Tsien, 1981; 1989). The indicators with these groups are usually referred to as the "AM" forms.

There are now a wide variety of pH and Ca^{2+} fluorescent indicators (see Tsien, 1989) being used. Recently, Tsien and collaborators (Minta and Tsien, 1989; Harootunian et al., 1989) also developed indicators for Na^+ ions. The currently used compound is the so-called sodium-binding benzofuran isophthalate (SBFI); see the molecule structure illustrated to the right. Basically, the molecule is made up of a central ring and a crown ether, with the two nitrogens attached to heterocyclic chains. The central ring has the appropriate size to preferentially bind Na^+ and, hence, provides the molecule with its Na^+-binding properties. The two heterocyclic chains are chromophores whose fluorescence properties depend on the binding of Na^+ to the



molecule, making it an effective Na^+-sensitive fluorescent indicator. Note the two carboxyl groups on each chromophore chain, which are mainly in the charged form at physiological pH (the pKa's are about 5.5 and 6.1 at 22°C, see Minta and Tsien, 1989). Thus, these carboxyl groups make the molecule heavily charged and, hence, membrane-impermeant. The membrane-permeant form of SBFI (SBFI-AM) would be constituted by the attachment of acetoxymethyl ester to the four groups. The basic properties of SBFI (determined at 22°C) have further been described by Minta and Tsien (1989). In solutions, the dye binds to Na^+ ions with a dissociation constant, k_d, of 7.4 mM. When measured in mixtures of Na^+ and K^+ such that their total concentration is kept constant at 135 mM, the apparent k_d became equal to 18 mM. This is due to the fact that SBFI can also bind to K^+ ions, with a selectivity of Na^+ over K^+ of about 20. This relatively high selectivity over K^+, together with an apparent dissociation constant in the physiological range of intracellular Na^+, combine to make this dye an appropriate Na^+ probe. In that regard, it is also important to note that Na^+ binding to the dye is practically insensitive, at least in their physiological range, to pH, Ca^{2+} and Mg^{2+} (Minta and Tsien, 1989; see also Haigney et al., 1991). The fluorescence properties of SBFI are such that its maximal emission intensity occurs around 520 nm, while the peak intensity of its excitation spectrum is in the range of 340-380 nm. Also of importance is the fact that the excitation spectrum shows a left shift of the peak intensity with increasing Na^+ concentrations. This allows the performance of quantitative determinations of Na^+ concentrations independent of the dye concentration, by measuring the ratio of emission intensity from two excitation

wavelengths. This ratio approach, initially developed for the Ca^{2+}-sensitive indicator FURA-2 (Grynkiewicz et al., 1985), proved to be extremely useful for intracellular ion measurements.

So far, only a limited number of studies using SBFI in cardiac cells have been published. With the exception of two studies on cultured embryonic cells (Ahlemeyer et al., 1992a; 1992b), experiments were performed on ventricular myocytes isolated from adult guinea-pig (Satoh et al., 1991; Harrison et al., 1992) or rat heart (Frampton et al., 1991; Lee and Levi, 1991; Donoso et al., 1992; Haigney et al., 1992; Harrison et al., 1992; Borzak et al., 1992). In all instances, the membrane-permeant SBFI-AM was used to load the myocytes. Overall, these studies showed that the dye can be effectively used to monitor changes of $[Na^+]_i$, with values comparable to what has been described previously. There are noteworthy observations that point to potentially important limitations for the quantitative determination of $[Na^+]_i$ with this approach, however. First, the properties of the dye appear to change markedly in the intracellular milieu (e.g. Ahlemeyer et al., 1992a; Donoso et al., 1992; Borzak et al., 1992), thereby necessitating the careful intracellular calibration of the dye. Secondly, it was found that as much as 50% of the dye could be distributed in the intracellular organelles rather than the cytosol (Donoso et al., 1992; Borzak et al., 1992), thereby giving possible erroneous measurements of intracellular free-Na^+ concentration. Finally, and most importantly, it was found (Haigney et al., 1992) that a substantial portion of SBFI-AM was not hydrolysed into the acid form, which can also lead to artifactual measurements. This latter problem

is in fact the major drawback of using the AM forms of all fluorescent indicators, as amply demonstrated with the use of Ca^{2+}-sensitive dyes (see Morgan, 1993). Partial deesterification of SBFI-AM is also likely responsible for the mentioned compartmentalization of the dye and may explain, at least in part, the apparent alterations of the dye properties when measured intracellularly. As discussed by Morgan (1993), the only way to circumvent these problems is through direct injection of the acid forms into the cells. This approach has yet to be used for SBFI, however, and its development and assessment will constitute the first objective of this research project.

PHARMACOLOGICAL TOOLS USED IN THIS STUDY

The following chemicals and pharmacological agents were employed as tools in this study. The relevant characteristics and properties, and the rationale for their use are discussed below.

IONOPHORES

Two ionophores, gramicidin and amphotericin B, were used to facilitate the movement of monovalent ions across the cell membrane, rendering the myocyte hyperpermeable, to allow the cell to achieve an equal distribution of intracellular and extracellular ions during the intracellular calibration of SBFI.

Gramicidin was used because it is highly conductive and selective to monovalent cations, (Finkelstein and Andersen, 1981; Harootunian et al., 1989). As amphotericin B is not totally selective to cations (Pressman, 1976; Carrasquer et al., 1989), it was chosen, in addition to gramicidin, because it facilitates the movement of positively *and* negatively charged ions, such as Cl^-, across the cell membrane. It was used here in an attempt to reach equal intracellular and extracellular concentrations of anions. Amphotericin was also used because it has a greater conductance for Na^+ than K^+ (Carrasquer et al., 1989), as opposed to gramicidin which has a slightly higher selectivity for K^+ than Na^+ (Pressman, 1976).

Gramicidin was also used in experiments focusing on studying the effects of isoproterenol on the Na-K pump. In an attempt to increase the $[Na^+]_i$ in these experiments,

low concentrations of gramicidin (<30nM) were used to produce transmembrane pores, facilitating the inward movement of Na^+ across the cell membrane.

Ca^{2+} CHELATORS

The Ca_{2+} chelator EGTA (Blinks, 1991) was used to remove extracellular Ca_{2+} during the intracellular calibration of SBFI, so as to inactivate the Na—Ca exchanger, and prevent cell contracture. EGTA was also used to assess the impact of the removal of extracellular Ca^{2+} on the effects of isoproterenol on the Na—K pump. EGTA was chosen because it is very selective to Ca^{2+}, (Tsien, 1980; Blinks, 1991). However, EGTA is highly sensitive to pH changes within the physiological range of pH (Martell and Smith, 1974), and also has a relatively slow reaction time for the uptake and release of Ca^{2+} (Hellan and Podolsky, 1969). Another Ca^{2+} chelator, BAPTA, is not sensitive to changes in pH, and has a relatively faster reaction time than EGTA (Tsien, 1980; Harrison and Bers, 1987; Blinks, 1991). The availability of the membrane permeant form of BAPTA (BAPTA—AM) made it the appropriate chelator to use for the removal of Ca^{2+} from the intracellular milieu, in order to investigate the hypothesis that intracellular Ca^{2+} is involved in the stimulation of the Na—K pump by isoproterenol.

STROPHANTHIDIN

Strophanthidin, like other cardiac glycosides, inhibits Na^+-K^+ ATPase. It was chosen for this study because its effect is specific to Na^+-K^+ pump, and is rapidly reversible (see Eisner and Smith, 1991).

RYANODINE

While studying the effects of isoproterenol on the Na—K pump in the presence of gramicidin, cell instability and spontaneous contractions occurred, resulting in mechanical injury to the cell attached to the microelectrode. These oscillations could be attenuated, however, by the use of ryanodine. It is well known that this alkaloid impairs Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (see Lytton and MacLennan, 1991), thereby inhibiting spontaneous contractions. As such, ryanodine was used for this purpose, and not for the depletion of intracellular stores of Ca^{2+}, in those experiments involving the combined use of gramicidin and isoproterenol.

ANTI—OXIDANTS

Since catecholamines are known to auto-oxidize (Taylor and Shappell, 1992) all the solutions containing isoproterenol were supplemented with 20 μM EDTA (Furchgott,1955) and 0.1 μM of reduced glutathione (Baillie and Slatter, 1991) to prevent oxidation of the ß-agonist. The anti-oxidants were also added to all the control solutions which contained gramicidin without isoproterenol, in order to ensure that isoproterenol was the only variable introduced.

OBJECTIVES

The overall objective of this research is to study the active Na^+ efflux in ventricular myocytes isolated from adult rabbit heart and its regulation by ß-adrenergic receptors. As mentioned above, several studies have indicated that the Na-K pump activity appears to be modulated by ß-adrenergic agonists. The mechanisms underlying this stimulation remain to be clearly understood, however, although the involvement of intracellular Ca^{2+} has recently been suggested. These mechanisms should be better understood with an experimental approach allowing the simultaneous measurements of intracellular Na^+ concentration and transmembrane ion current in single isolate cardiac myocytes.

Accordingly, the specific objectives of this research project are:

1) To develop a technique that will allow the micro-injection of the Na^+-sensitive fluorescent indicator SBFI in the cardiac myocytes, and to characterize the fluorescent properties of the dye measured under those conditions.

2) To develop an approach to study the mechanisms involved in the regulation of intracellular Na^+ through the simultaneous measurement of $[Na^+]_i$ and transmembrane current under voltage-clamp conditions.

3) To verify that isoproterenol directly stimulates the Na-K pump in voltage-clamped isolated single ventricular cardiac myocytes in rabbit.

4) To test the hypothesis that Ca^{2+} is involved in the ß-adrenergic regulation of the active Na^+ efflux.

METHODS

ISOLATION OF CARDIAC MYOCYTES

Cardiac myocytes were isolated from adult rabbit ventricular septum by a procedure similar to that described previously (Désilets and Baumgarten, 1986 a,b). Animals (2 - 3 Kg) were sacrificed by cervical dislocation. The hearts were quickly removed, mounted on a Langendorff apparatus and retrogradely perfused through the aorta with oxygenated modified Tyrode's solution (Table 1, Solution A). Perfusion pressure was set by the height of the column at about 100 cm H_2O. The solution contained 2 mM $CaCl_2$, thereby allowing spontaneous contractions of the heart and washout of blood. After 4 min. of perfusion, the heart was transferred to a second column containing a nominally Ca^{2+}-free modified Tyrode's solution (Table 1, Solution B) and perfused for another 4 min. The perfusate was then replaced with 50 ml of the collagenase containing solution (Table 1, Solution C) and this solution was recirculated for approximately 12-16 min. Monitored pressure usually fell to zero after about 2 to 4 min. At that time, $CaCl_2$ concentration was brought up to a final value of 50 μM. Collagenase was then flushed out by perfusing the heart for 2 min with the modified solution B, containing the $CaCl_2$ concentration of 50 μM. The heart was then removed from the apparatus, and the ventricular septum was isolated and cut into small pieces. These pieces were gently shaken in 20 ml of the

Table 1: Solutions used for Myocyte Isolation and SBFI Calibration

Upper Panel (A,B,C): Solutions used for myocyte isolation.

Lower Panel (D,E,F): Solutions used for calibration of SBFI.

All solutions contained 5 mM HEPES.

For the extracelllular calibration of SBFI (solution D) the [Na^+] varied ; 0, 0.3, 1.0, 2.0, 4.0, 7.5, 15, 30, 61 and 138 mM with the corresponding [K^+] to make the total concentration for the two equal to 150 mM.

For the intracellular calibration of SBFI (solution E) the [Na^+] varied; 0, 3, 6, 12, 24, 50, and 100 mM with the corresponding [K^+] to make the total concentration for the two equal to 150 mM.

TABLE 1

	A	B	C
Chemical (mM)			
$CaCl_2$	2.0	----	25 μM
Collagenase	----	----	1 mg/ml
KCl	5.0	5.0	5.0
NaCl	145	145	145
$MgCl_2$	1.2	1.2	1.2
D-Glucose	10	10	10
pH	7.4	7.4	7.4

	D	E	F
Chemical (mM)			
$CaCl_2$	0.36	0.36	0.8
EGTA	1.0	2.0	2.0
NaCl + KCl	150	150	145 + 5
$MgCl_2$	2.5	2.8	2.4
D-Glucose	---	---	10
Chemical (μM)			
SBFI	5.0	---	---
Gramicidin	---	4.0	4.0
Amphotericin B	---	1.0	1.0
Strophanthidin	---	500	---
pH	7.2	7.2	7.4

modified B solution containing 50 μM $CaCl_2$ until the complete dissociation of the tissue. The resulting cell suspension was filtered through a 200-μm nylon mesh. The cells were left to sediment by gravity and the supernatant replaced with fresh modified Tyrode's solution (Table 1, solution A). The cells were kept at 37°C in this solution, unless otherwise specified, and used within 8 hours of isolation. The percentage of rod-shaped cells with clear striation which were not swollen (thereby considered viable for the purposes of this study) varied extensively between experiments (range of 30 to 70%). Morphological assessment revealed that this fraction of viable cells remained constant during the 8 hour period. The viability of the cells was ensured by regularly replacing the incubation solution.

ELECTRICAL AND FLUORESCENCE RECORDINGS

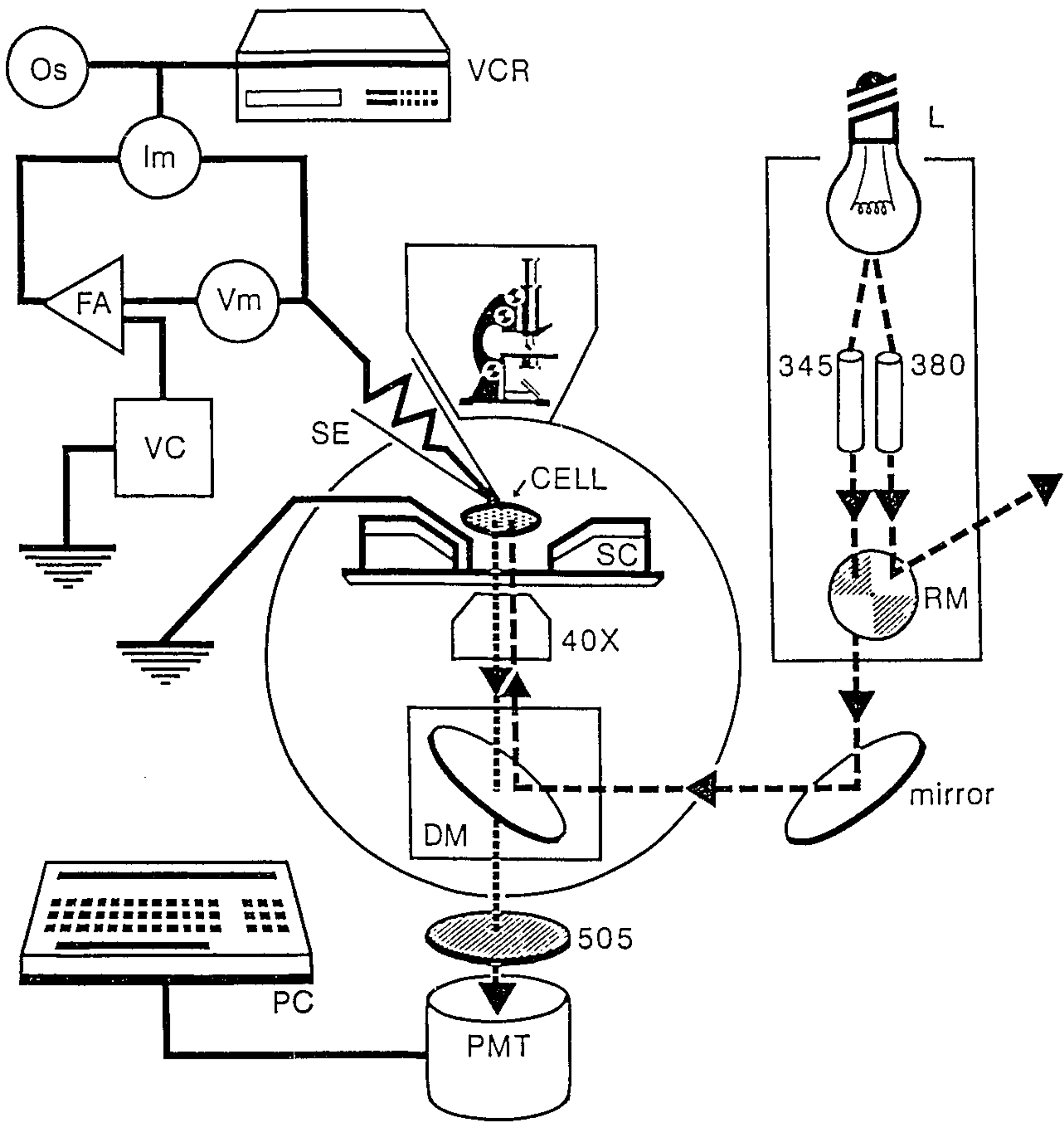
The procedures to record membrane electrical activity and cell fluorescence were done in a fashion similar to that described elsewhere (Désilets et al. 1989, Eley et al. 1991). The set-up is schematically represented in Figure 1. Myocytes were put in a 0.3 ml glass-bottom superfusion chamber which was placed on the stage of an epifluorescent inverted microscope (Nikon Diaphot-DM). This microscope was placed on an air-cushioned table which prevented transmission of vibration through the microelectrode which in turn, reduced mechanical injury to the myocytes and eliminated movement artifacts. The cells were allowed to adhere to the bottom of the chamber before starting the superfusion, with the flow rate maintained at approximately 5 ml/min. The temperature was kept constant at 37±0.5°C by heating the solution at the inlet of the chamber. Switching between solutions was performed using electrical valves (General Valves) with a total dead space of less than 0.5 ml.

The injection of fluorescent indicators and the recording of electrical activity, as described below, was performed by impaling each cell with a small suction pipette filled with a solution of the following composition (in mM): K aspartate, 95; KCl, 20; KH_2PO_4, 1; K_2ATP, 2.5; MgATP, 2.5; K_2-phosphocreatine, 2.5; taurine, 10; HEPES, 5; EGTA, 0.5; pH adjusted to 7.2 with KOH. The composition of this solution was chosen to mimic the intracellular environment. Impalements were obtained by applying gentle suction in the pipette upon contact of the tip with the cell membrane, while monitoring the electrode

FIGURE 1. Diagram of the setup used for simultaneous recordings of cell fluorescence and transmembrane current in isolated cardiac myocytes.

Single cells were deposited on the bottom of a superfusion chamber (SC) placed on the stage of an epifluorescent microscope equipped with a 40X objective. Excitation light was provided by a Xenon lamp (L) and initially split into two parallel beams filtered at 345 and 380 nm. The light beams were directed alternately towards the microscope dichroic mirror (DM) through a rotating mirror (RM). Emission light was captured by a photomultiplier tube (PMT) after being filtered at 505 nm. The analog signal was then digitized and processed by computer (PC). Intracellular loading of the fluorescent indicator was made by injection from a small suction electrode (SE) which was also used for whole-cell patch clamp recording. Membrane potential, V_m, was clamped through a feedback amplifier (FA) connected to a voltage command generator (VC). The resulting transmembrane current, I_m, was recorded on an oscilloscope (Os) and stored on a modified video cassette recorder (VCR) for subsequent computer analysis.

FIGURE 1



voltage and resistance. Access to the cell interior at the tip of the electrode was monitored by a sudden shift of the electrode voltage to about -80 mV. Only cells with stable membrane potentials were subsequently studied.

A. Voltage-clamp:

Measurements of transmembrane ion currents were performed according to procedures similar to the whole-cell patch-clamp technique (Hamill et al. 1981), except that the suction electrodes used here had relatively small tips (DC resistance of ~60 MΩ) in order to minimize intracellular dialysis. Furthermore, recordings of membrane potential and currents were performed by the discontinuous voltage-clamp technique (Finkel and Redman, 1984) whereby the electrode is switched at high frequency (about 2 kHz) from a voltage-recording to a current-passing mode. The advantage of this approach is that it bypasses the voltage drop across the electrode resistance, thereby eliminating the need to perform resistance compensation for those relatively resistive electrodes.

The electrical ground was provided using a 3 M KCl bridge in contact with the bath solution. Current injection by the voltage-clamp amplifier (Axon Instruments, Axoclamp-2A) was controlled by a timer/stimulator (Winston Electronics, A-65). The current and voltage signals were recorded by oscilloscopes and stored on videotapes (Vetter, 420-B) for off-line analyses.

Digital transfer of these data to the computer was performed at a sampling rate of

200 Hz after analog filtering at a corner frequency of 100 Hz. Unless otherwise specified, currents were digitally filtered at a frequency of 5 Hz before analysis. In a previous study performed by another researcher from the same laboratory (Désilets and Baumgarten, 1986a), a 10 mV junction potential difference developed upon impalement of rabbit isolated ventricular myocytes under conditions similar to those of the present research project. Therefore, a junction potential difference correction of 10 mV was applied prior to the analysis of the electrical data. These analyses of the data and their plotting were done using an integrated software application (Keithly Instruments, ASYST).

B. Spectrofluorometric determinations of intracellular Na^+ concentration.

Determination of $[Na^+]_i$ was performed using the Na^+-sensitive fluorescent indicator SBFI (Minta and Tsien, 1989) and measuring the ratio of fluorescence intensities at two different excitation wavelengths. Cellular injection of the dye was done by iontophoresis. This method takes advantage of the fact that the indicator is negatively charged at neutral pH. The procedure consisted of including 1.5 mM SBFI (Molecular Probes, free acid form) in the suction pipette solution and applying a hyperpolarizing current (-1 nA) after accessing the interior of the cell. Injection was maintained until the fluorescence intensity reached values at least 4 times the background intensity, which usually took 2 - 4 minutes (see results). This iontophoresis technique was required because of the small size of the pipette tip, which greatly limited diffusion of SBFI into the cell.

The measurements were carried out by a spectrofluorometer (Spex Industries, Fluorolog CM system) connected to the epifluorescent inverted microscope (Figure 1). Excitation light was provided by a Xenon lamp. The light was split into two beams, each of which were filtered by separate monochrometers set at 345 and 380 nm. These two excitation wavelengths were chosen based on the properties of SBFI fluorescence intensity which was determined to be most sensitive to Na^+ concentrations at these two values (see Results). The two beams were directed in parallel towards an 8-blade rotating mirror causing rapid alternation between the two incoming beams. Single myocytes in the superfusion chamber were exposed to the resulting excitation light beam, which was focused on a cell through a 40X objective lens after being directed by 400 nm dichroic mirror. The emission light, filtered at 505 nm, was received by a photomultiplier tube, passed to a computer and digitally processed (Spex Industries, DM3000 software). Note that this computer was also controlling the speed of the rotating mirror, thereby allowing synchronized acquisition of the fluorescent light emitted at the two different excitation wavelengths. Unless otherwise specified, data were acquired at a sampling rate of 10 Hz and digitally filtered at 2 Hz. Measurements of fluorescence were made through an adjustable rectangular window set at about 300 μm^2 such that only a portion of the myocyte fluorescence was measured. This window was situated far enough from the site of impalement so as to avoid picking up the high intensity fluorescing light coming from the tip of the electrode. Background fluorescence at the two excitation wavelengths was measured before SBFI injection, and later subtracted from the respective SBFI

fluorescence signals.

Ratio calculations of fluorescence intensities, as well as further data analysis and digital filtering were performed using the ASYST software. This same software was used to synchronize the fluorescence signals with the previously described voltage-clamp recordings acquired from the video cassette recorder.

EXPERIMENTAL PROTOCOLS

A. Calibrations of SBFI Fluorescence

The fluorescent indicator SBFI was first prepared as a stock solution at a concentration of 1.5 mM in the electrode solution (composition described in the previous section). Two types of calibration of SBFI were performed; extracellular calibrations from SBFI-containing solutions without the presence of myocytes, and then intracellular calibrations from SBFI-loaded myocytes.

The extracellular calibrations were performed in the same superfusion bath as the one used for cell recordings. SBFI was dissolved in solutions of various Na^+ concentrations (Table 1, Solution D) to give a final concentration of 5 μM. These solutions were kept at 37°C and their fluorescence was measured from a volume of 0.3 ml put in the superfusion chamber.

The intracellular calibrations were carried out on SBFI-loaded myocytes in solutions similar to those used for extracellular calibrations, except that they also contained 4 μM gramicidin, 1 μM amphotericin B and 0.5 μM strophanthidin (Table 1, Solution E). The ionophores were added in order to render the cell membrane hyperpermeable to monovalent cations while strophanthidin was added to inhibit the Na-K pump and, hence, to allow passive distribution of Na^+ ions. In order to ensure maximum incorporation of the ionophores in the sarcolemma, cells were preincubated for at least 45 min prior to

impalement in a solution containing the ionophores (Table 1, Solution F). This preincubation, and the fluorescent measurements of SBFI-loaded myocytes, were both performed at 37°C. Solutions of varying [Na^+] were switched randomly, with an exposure time of from 5 to 10 min for each solution. Due to these prolonged exposures, the majority of the cells did not survive long enough to be studied in all 7 calibration solutions. For the purposes of this study, a cell which did not survive is defined as one which detached from the microelectrode, moved outside the fluorescent measurement window and/or did not maintain its initial rod-shaped morphological appearance. Furthermore, data were considered for analysis only when steady-state of fluorescence signals were clearly reached after changing the solution. Consequently, different concentrations of Na^+ were represented by mixed populations of cells on the calibration curve.

Both intracellular and extracellular calibrations were determined by plotting the ratios of light intensities measured at excitation wavelength of 345 nm over those at 380 nm as a function of Na^+ concentrations. Data were then fit with an equation derived from the classical calibration equation used for fluorescent indicators having dual excitation properties (Grynkiewicz et al., 1985), applied to our conditions. This equation is written as follows:

$$[Na^+]=C\frac{(R-R_{min})}{(R_{max}-R)} \qquad (1)$$

where R is the 345/380 ratio at a given Na^+ concentration, R_{min} and R_{max} are the ratios

obtained at 0 mM and saturating Na^+ concentrations respectively, and C is the apparent dissociation constant (k_{app}), which is the Na^+ concentration giving the ratio value at the mid point between R_{min} and R_{max}. Knowing the value of these constants, one can therefore determine Na^+ concentration at any given R value. However, SBFI fluorescence does not saturate at 150 mM Na^+ (see Results), so R_{max} cannot be measured unless the osmolarity is changed. To circumvent this problem, Equation 1 can be transformed by replacing R_{max} with another constant measured at a given Na^+ concentration, for instance, the R value measured at 100 mM Na^+, R_{100}.

Thus, at 100 mM Na^+, Equation 1 becomes:

$$[Na^+] = 100 = C\frac{(R_{100}-R_{min})}{(R_{max}-R_{100})} \quad (in\ mM) \qquad (2)$$

Isolating R_{max} from this equation and placing it in Equation 1, gives the following:

$$[Na^+] = \frac{100C(R-R_{min})}{100(R_{100}-R)+C(R_{100}-R_{min})} \quad (in\ mM) \qquad (3)$$

Equation 3 was used to fit the calibration curves by a least-square non-linear curve fitting procedure, the Gauss-Newton method, provided by the ASYST software.

B. Determination of the Effects of ISO on $[Na^+]_i$

Unless otherwise specified, the following procedures were performed in all the experimental protocols.

The cardiac myocytes were placed in the bath, and superfused with a flow rate of 5 ml/min with the appropriate modified Tyrode's solution. A myocyte from the group was chosen as the target cell, and the fluorescent measurement window was determined. In order to ensure that the cell received equal exposure from each of the two monochrometers, both were initially set at an excitation wavelength of 380 nm. The air-cushioned table was then balanced to provide equal intensities of emission light from both sources. The monochrometers were then reset to values of 345 and 380 nm respectively. The SBFI filled electrode was placed adjacent to the cell membrane, near the window, while staying outside the range of fluorescent measurement. The background fluorescence of the cell was then measured.

The cells were impaled, injected with the fluorescent indicator, voltage-clamped at the desired membrane potential, and then subjected to the specific experimental protocols described in sections B1 through B7 to follow.

Not all the cells survived all the steps of the protocol under which they were studied. For the purposes of this study, a cell which did not survive is defined as one which detached from the microelectrode, moved outside the fluorescent measurement window, did not maintain its initial rod-shaped morphological appearance, and/or could

no longer be voltage-clamped at the desired membrane potential.

Fluorescent measurements were taken for 60 sec before the addition of ISO, for 60 sec starting one minute after the introduction of ISO, and for 60 sec starting two and a half minutes after the washout of ISO. For simplicity, data were averaged around the midpoint of measurements and expressed as rate of change of $[Na^+]_i$ as measured 30 sec before exposure to isoproterenol (CONTROL), 90 sec after the addition of isoproterenol (ISO), and 180 sec after its removal (WASHOUT).

Since electrical events had a faster time course than fluorescent changes, electrical measurements were taken immediately before the addition of isoproterenol (CONTROL), 30 sec after its addition (ISO), and 150 sec after its removal (WASHOUT).

B.1 Under steady-state conditions:

The cardiac myocytes were superfused with a calcium containing Tyrode's solution similar to solution A in table 2 except that the Ca^{2+} in this case was 2 mM. Membrane potential was clamped at -80 mV, and allowed to stabilize for approximately 3 min. The cell was then exposed to 0.5 μM ISO for a period of about 3 min, after which the ISO was washed out. Fluorescent measurements were taken before ISO exposure, at 30, and 60 sec after its introduction.

B.2 The use of gramicidin to create a steady Na^+ influx:

The next set of experiments involved developing a protocol to assess the possibility of creating a steady Na^+ leak using the ionophore gramicidin and analyzing dose responses to establish an appropriate concentration.

Cardiac myocytes were superfused with the same solution as in steady-state conditions (Table 2, Solution A). The cells were impaled, injected and then voltage-clamped at -70 mV.

After stabilization, the cells were superfused with gramicidin (5-1000 nM). To test the reversibility of the effects of gramicidin, the cells that survived were then washed with Solution A (Table 2).

Table 2: **Solutions used in Experiments to Study the Effects of ISO on $[Na^+]_i$ in Isolated Cardiac Myocytes**

Upper Panel (A,B,C,D,E,F): Solutions used to study the effects of ISO on $[Na^+]_i$ in the presence (Ca^{2+}) or absence (EGTA) of extra cellular Ca^{2+}.

Lower Panel (G,H,I,J,K,L): Solutions used to study the effects of ISO on $[Na^+]_i$ in BAPTA-AM pre-incubated cells (BAPTA) and solutions used to study the effects of ISO in presense if strophanthidin (STR).

All solutions contained 145 mM NaCl, 5 mM KCl, 5mM HEPES, 1.8mM NaH_2PO_4, and 10 mM D-Glucose at pH = 7.4

TABLE 2

	Ca^{+2}			EGTA		
	A	B	C	D	E	F
Chemical (mM)						
$CaCl_2$	0.8	0.8	0.8	---	---	---
EGTA	---	---	---	0.5	0.5	0.5
$MgCl_2$	2.4	2.4	2.4	3.2	3.2	3.2
Chemical (μM)						
Ryanodine	---	0.1	0.1	---	0.1	0.1
Gramicidin	---	0.01	0.01	---	0.03	0.03
ISO	---	---	0.5	---	---	0.5
EDTA	---	20	20	---	20	20
glutathione	---	0.1	0.1	---	0.1	0.1

	BAPTA		STR			
	G	H	I	J	K	L
Chemical (mM)						
$CaCl_2$	---	---	0.8	0.8	---	---
EGTA	0.5	0.5	---	---	0.5	0.5
$MgCl_2$	3.2	3.2	2.4	2.4	3.2	3.2
Chemical (μM)						
Ryanodine	---	---	0.1	0.1	0.1	0.1
Gramicidin	0.03	0.03	0.01	0.01	0.03	0.03
Strophanthidin	---	---	100	100	100	100
ISO	---	0.5	---	0.5	---	0.5
EDTA	20	20	20	20	20	20
glutathione	0.1	0.1	0.1	0.1	0.1	0.1

B.3 In the presence of external Ca^{2+}:

Initially the myocytes were superfused by a calcium containing solution (Table 2, Solution A) and voltage-clamped at -70 mV. After stabilization the cells were exposed to a gramicidin solution (Table 2, Solution B) until the current shifted to a negative direction (occurring between 3-5 min). Then, the cells were superfused with ISO (Table 2, Solution C) for approximately 2-3 min. Solution B was then used to wash out ISO.

B.4 In the absence of external Ca^{2+}:

The myocytes were treated in the same way as described in section B.3, except that they were initially superfused by an EGTA containing solution (Table 2, Solution D). The cells were then treated with solution E followed by solution F, which was washed out later by solution E (from Table 2) in a similar way as described in the previous section.

B.5 In the presence of strophanthidin and Ca^{2+}

In this set of experiments, only transmembrane current measurements were taken. Fluorescent measurements, which require long time periods, were difficult to acquire because strophanthidin inhibits the Na-K pump, and as a result the cell becomes loaded with Na^{+} which, in turn, inhibits Ca^{2+} efflux through the Na-Ca exchanger (Wier, 1991).

This causes a state of contracture in the myocytes, which made the recording of fast changes to the holding current the only possible means of measurement.

Cells were superfused by solution A, table 2, impaled, and then voltage-clamped at -70 mV. The cells were then superfused with the gramicidin containing solution B, until the current shifted to a negative direction (occurring between 3-5 min). The next step was to expose the cell to 100 μM of strophanthidin (Table 2, Solution I) for approximately 15 - 20 sec. ISO was added (Table 2, Solution J) for a 30 sec pulse and then washed out with solution I for at least 30 sec. The strophanthidin was then washed out using solution B. Some cells were exposed to a 30 sec pulse of ISO (Table 2, Solution C), either before the addition or after the wash out of strophanthidin.

Electrical measurements were taken immediately before the addition of strophanthidin (STR), 15 sec after its introduction (CONTROL), 30 sec after the addition of isoproterenol (ISO) and 30 sec after washout of the isoproterenol (WASHOUT).

B.6 In the presence of strophanthidin and EGTA

Initially the myocytes were superfused by an EGTA containing solution (Table 2, Solution D), impaled, injected with SBFI and voltage-clamped at -70 mV. After stabilization the cells were exposed to a gramicidin containing solution (Table 2, Solution E) until the current shifted to a negative direction. Then, the cells were superfused with strophanthidin (Table 2, Solution K). The cells were then exposed to ISO for 2 - 3 min

(Table 2, Solution L) and then washed out with solution K. The strophanthidin was washed out using solution E.

B.7 In myocytes loaded with BAPTA-AM

Myocytes were incubated for 2 - 3 hours before SBFI injection in a Ca^{2+}-free modified Tyrode's solution containing 0.5 mM EGTA and 25 μM BAPTA-AM, then the BAPTA containing solution was replaced with another modified Tyrode's containing 0.5 mM EGTA (Table 2, Solution D).

After BAPTA wash out, this protocol was conducted in the same manner as in B.4, with the following two differences. First, since, for undetermined reasons the BAPTA loaded cells did not tolerate ryanodine, (i.e. lost their rod-shape and striation and became very difficult to impale), solutions used in this protocol were ryanodine-free. Secondly, as some cells were depolarized, the command voltage for the voltage-clamp was decided according to the membrane potential at the time of impalement.

The myocytes were placed in the superfusion chamber, superfused with solution D, impaled and injected with SBFI. Depending upon the cell membrane potential at the time of impalement, the cells were voltage-clamped at either -10 or -70 mV. After stabilization the cells were exposed to a gramicidin solution (Table 2, Solution G) until the current shifted to a negative direction. Then, the cells were superfused with ISO (Table 2, Solution H) for 2 - 3 min. The ISO was then washed out with solution G.

STATISTICAL ANALYSIS

There were two principal variables under consideration in this project; transmembrane current (in pA) and fluorescence measurements related to Na^+ concentration. The secondary variables, such as changes in currents and in the sodium ion influx, were derived from these two principal variables.

The two-way analysis of variance (ANOVA) for repeated measures (see Schutz and Gessaroli, 1987), with the source animal as a grouping factor between cells, was used to compare different treatments within cells studied under the same experimental protocol. This ANOVA was used because experimental conditions made it difficult to obtain measurements from an equal number of cells from each source animal.

In the repeated measures analysis, only those cells from which a measurement was possible at each time point were included in the comparison - this accounts for the discrepancy in the number of cells reported in this type of test, as compared to the number reported when comparing the values of change associated with a specific experimental time point between different experimental groups. Whenever three or more time points were tested simultaneously, significant ANOVAs were followed up by paired *t*-tests for comparisons of interest (those determined a priori). The significance level was adjusted for multiple comparisons using the Bonferroni method (Glantz, 1987).

Whenever the outcome of the analysis showed an interaction with, or a significant effect for, the source animal, the values of the measurements for individual cells were

averaged for each source animal, and one-way ANOVAs for repeated measures (see Glantz, 1987) were performed on the averaged data. The results of the averaged data generally confirmed the results derived from individual cells. The only difference was that the p-values of the averaged data tended to be higher (due to decreased sample size), causing some results that were statistically significant at the individual cells level to trend toward difference when averaged data were used. Only the results of tests done on individual cells are presented.

For the comparison of groups of cells under different experimental conditions, the one-way ANOVA for independent measures (see Stevens, 1986) was used. In group comparisons where the data sets did not meet the ANOVA assumptions, the nonparametric statistical test; Mann-Whitney U test was performed(see Glantz, 1987).

All tests were considered statistically significant when $p<0.05$. All values are expressed as means±SEM.

The principal statistical package for personal computers used for these analyses is Systat version 5.03 (Systat Inc.).

RESULTS

PROPERTIES OF SBFI IN EXTRACELLULAR SOLUTIONS

The fluorescence properties of SBFI were first characterized in a range of solutions containing 5 μM SBFI and various concentrations of Na^+ and K^+, with the total concentration maintained at 150 mM (composition given in Methods Section). These measurements were performed at 37°C in the same superfusion chamber and with the same optical system used for the intracellular recordings.

Figure 2 (panel A) illustrates the excitation spectrum of SBFI determined under the above conditions. As expected, increasing Na^+ concentration caused an augmentation of the fluorescence intensity below 370 nm, as well as a left shift of the peak value from 352 nm in 0 mM Na^+ to 346 nm in 138 Na^+. Beyond the isosbestic point, observed at 369 nm, increasing $[Na^+]$ caused a decrease of the light intensity. This cross-over phenomenon allows for a calibration procedure, independent of the SBFI concentration, by taking the ratio of fluorescence intensities measured at two different excitation wavelengths. Figure 2 (Panel B) shows the calibration curve, derived from panel A, in which the ratios between the values measured at 345 nm and those measured at 380 nm are plotted as a function of their respective $[Na^+]$. These two wavelengths were chosen to give a maximal variation, and hence, sensitivity, of the ratio values when changing $[Na^+]$. In this case, the

FIGURE 2. Calibration of SBFI in the extracellular milieu.

Panel A: Excitation spectra of SBFI (5 μM) dissolved in solutions containing Na^+ concentrations of (in mM): 0, 0.3, 1, 2, 4, 7.5, 15, 30, 61 and 138. Increasing Na^+ concentrations caused an increase of the peak fluorescence intensities. The curves were drawn after subtracting background fluorescence measured from solutions containing no SBFI. Intensity units are arbitrary.

Panel B: Resulting calibration curve for the ratios of the fluorescence intensities measured at excitation wavelength of 345 nm to the corresponding ones measured at 380 nm. Note the logarithmic scale on the Na^+ concentration axis. The solid curve was fitted according to the following calibration equation (see Methods):

$$RATIO = \{([Na^+]C(R_{100}-R_{min})+100([Na^+]R_{100}+CR_{min})\}/100([Na^+]+C)$$

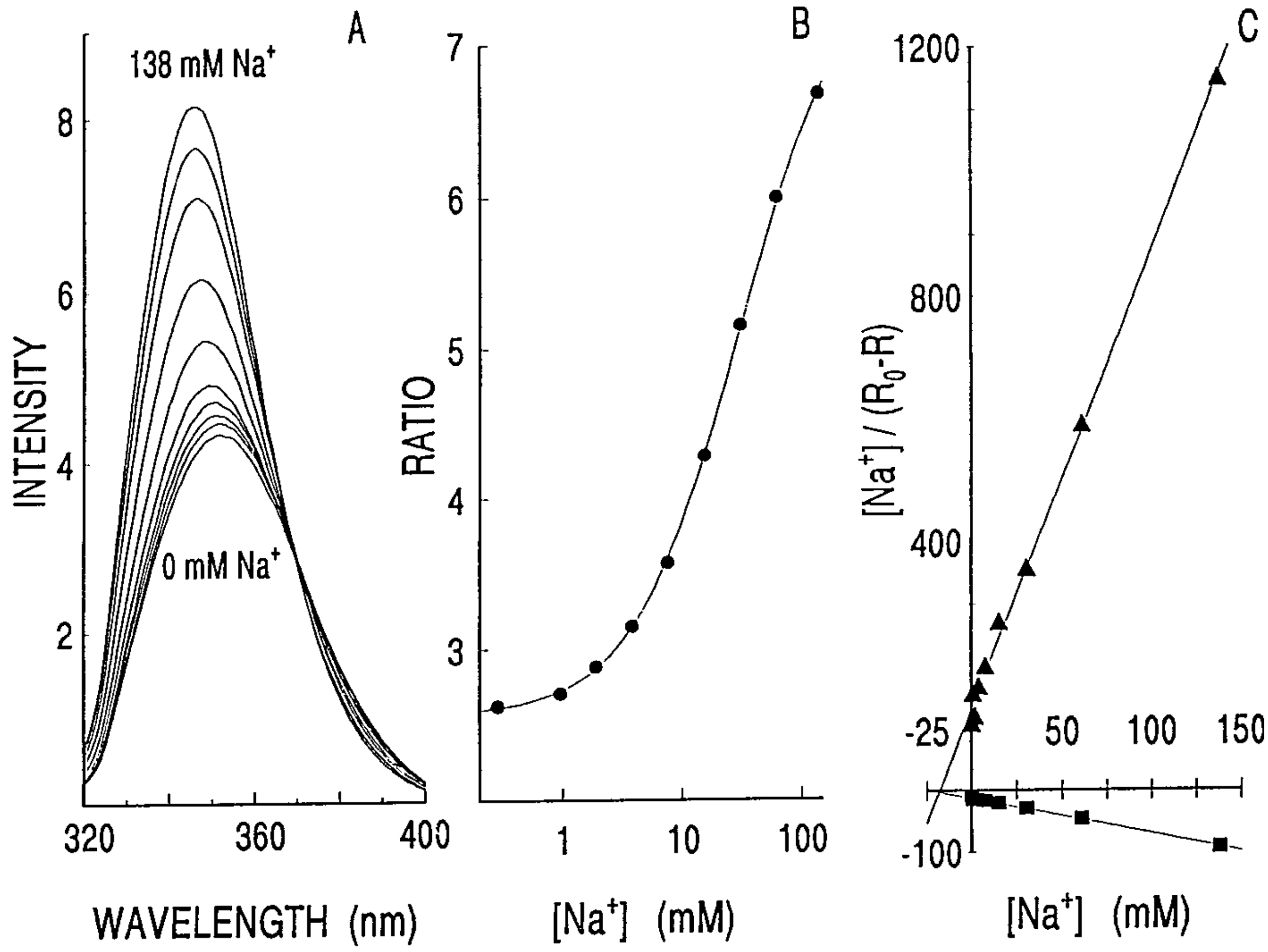
with C=28.5 mM; R_{min}=2.56; R_{100}=6.44

Panel C: Determination of the apparent dissociation constant, k_{app}, of SBFI for Na^+. This determination was obtained from the intensity values measured at 345 nm (squares) and 380 nm (triangles). The plots are represented as a Hanes transformation of the Michaelis equation, as modified from Donoso et al. (1992):

$$R-R_0 = (R_\infty-R_0)[Na^+]/([Na^+]+k_{app})$$

where R is the ratio of the fluorescence intensity measured at a given wavelength (345 or 380 nm) over that measured at the isosbestic point (369 nm); R_0 the ratio value measured in the absence of Na^+; R_∞ the ratio value at the hypothetical saturating concentrations of Na^+; and k_{app} the apparent dissociation constant ($-k_{app}$ is given as the abscissa intercept on the Hanes plot). Thus, k_{app} was found to be equal to 22.6 and 17.8 mM for the data at 345 and 380 nm, respectively.

FIGURE 2



ratio varied between 2.56 in nominally Na^+-free solution to 6.67 at 138 mM Na^+. In Figure 2 (Panel B), the data were fitted with equation 3 described in the Methods Section. This yielded a value for the constant C of 28.5 mM, which represents the Na^+ concentration that will result in a ratio value corresponding to the mid-point between R_{min} and R_{max}. For future reference, this will be termed the mid-ratio concentration. The excitation scan given in panel A of Figure 2 could also be used to determine the apparent dissociation constant, k_{app}, for the complex Na-SBFI, assuming a simple Michaelis behaviour (see Donoso et al., 1992). This is shown in panel C of Figure 2 which represents a Hanes plot of the values measured both at 345 and 380 nm. Thus, the k_{app} values obtained from the curve fitting were found to be 22.6 and 17.8 mM for the points measured at 345 and 380 nm, respectively.

Experiments were also performed to determine whether SBFI properties could be affected by the major compounds used in this study. Thus, SBFI fluorescence was also measured in the presence of gramicidin (4 µM), amphotericin (1 µM), strophanthidin (0.5 mM) and ryanodine (1 µM). For all cases, no change of the fluorescence ratio of 345 to 380 nm could be detected in solutions containing either zero or 100 mM Na^+.

INTRACELLULAR INJECTION AND CALIBRATION OF SBFI

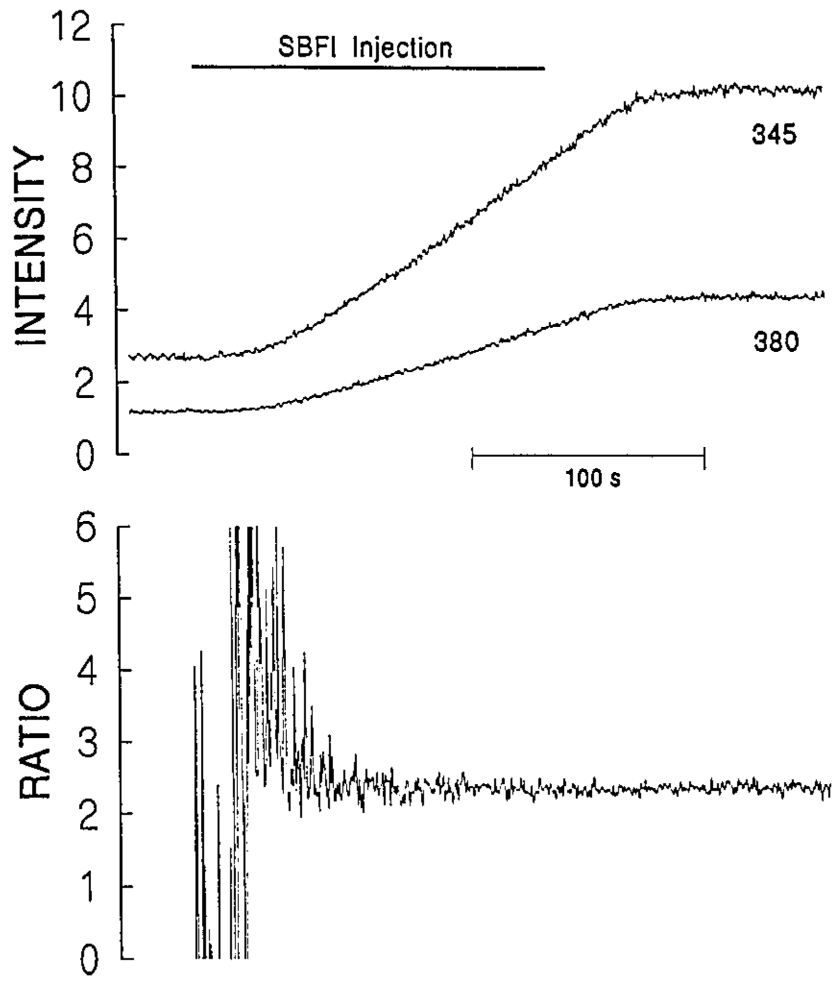
A typical recording of cell fluorescence during iontophoretic injection of SBFI is presented in Figure 3. The upper panel shows the change of fluorescence intensities at 345 and 380 nm, as observed when a 1 nA hyperpolarizing current is applied to the cell. Note that the background fluorescence did not change prior to the current injection, thereby demonstrating the absence of leakage of SBFI from the pipette into the cell. The discontinuation of the negative current resulted in steady-state levels of light intensities, which also indicated that the cell does not lose its SBFI after the injection. In 94% of the cells injected with the indicator (98 of 104), the membrane potential values before and after the injection were identical. The average membrane potential for cells superfused in Tyrode's solution containing 0.8 mM Ca^{2+} was -77.1±0.1 mV (n=60).

Figure 3 also shows the ratio of the intensities (lower panel), which was determined after subtracting the respective background values. The large fluctuations initially observed are caused by the relatively small amount of indicator injected during that time. More importantly, one can observe the rapid stabilization of the trace toward a steady level. This readily demonstrates that ratio determination is independent of SBFI concentration, and indicates that the intracellular Na^+ level remained constant during the injection. On average, the ratio value measured at the end of SBFI loading was equal to 1.89±0.043 (n=60). This value is lower than the minimal ratio (2.56) obtained from extracellular calibrations (see Figure 2). This observation directly demonstrates the need

FIGURE 3. Typical example of fluorescence recording during iontophoretic injection of SBFI. After impalement of the myocyte with the suction electrode, a negative current of 1 nA was applied to induce intracellular injection of SBFI during the period indicated by the horizontal bar. Upper panel: The tracings represent the changes of emission intensities (in arbitrary units) as induced by excitation light beams with wavelengths of 345 and 380 nm.

Lower panel: Ratio of the light intensity measured at 345 nm over that measured at 380 nm after background subtraction. Levels of background fluorescence were measured before SBFI injection. They correspond to the first 20 sec of the tracings in the upper panel.

FIGURE 3



to carry out intracellular calibrations, rather than using those obtained in bath solutions.

This intracellular calibration was performed by exposing the SBFI-loaded myocytes to solutions containing various concentrations of NaCl. These solutions were similar to those used for extracellular calibration, except that they also contained the ionophores gramicidin (4 μM) and amphotericin (1 μM), as well as a saturating concentration (500 μM) of strophanthidin. Figure 4 illustrates an experiment where a cell was exposed to three different concentrations of Na^+. As expected, changing [Na^+] had opposing effects on the fluorescence intensities measured at 345 and 380 nm (upper panel), such that the changes of their ratio values paralleled those of [Na^+](lower panel), with steady-state values being reached within 5 min after changing [Na^+] in the superfusing solution. It should be mentioned that exposure of the myocytes to the solutions containing gramicidin and amphotericin rapidly resulted in membrane depolarisation; the membrane potential reaching a mean value of −7.8±2.4 mV (n=8), with 100 mM extracellular Na^+. This value became slightly more positive in solutions containing low Na^+ concentrations, the steady-state value being equal to −4.6±1.8 mV (n=7) for cells superfused in zero mM Na^+. It should also be noted that all cells demonstrated a tendency to swell after the prolonged exposure to the calibration solutions.

Figure 5 represents the ratio values plotted as a function of their corresponding [Na^+]. The fitting of these data points resulted in a mid-ratio concentration value of 158 mM, with the minimum ratio equal to 1.44. These values are very different from those determined from calibration of SBFI in the bath, (28.5 mM and 2.56, respectively), as can

FIGURE 4. Typical example of fluorescence recording for intracellular calibration.

The myocyte was first incubated for 45 min in a modified Tyrode's solution containing 2 mM EGTA, 4 μM gramicidin and 1 μM amphotericin and then impaled for SBFI injection. Recording started after about 5 min in a calibration solution containing 24 mM Na^+ followed by exposures to 0 and 50 mM Na^+. In addition to the ionophores, these 3 calibration solutions also contained 500 μM ouabain.

Upper panel: Changes of fluorescence intensities at 345 and 380 nm. The intensity scale is in arbitrary units.

Lower panel: Corresponding ratio of the fluorescence intensities obtained after background substraction.

FIGURE 4

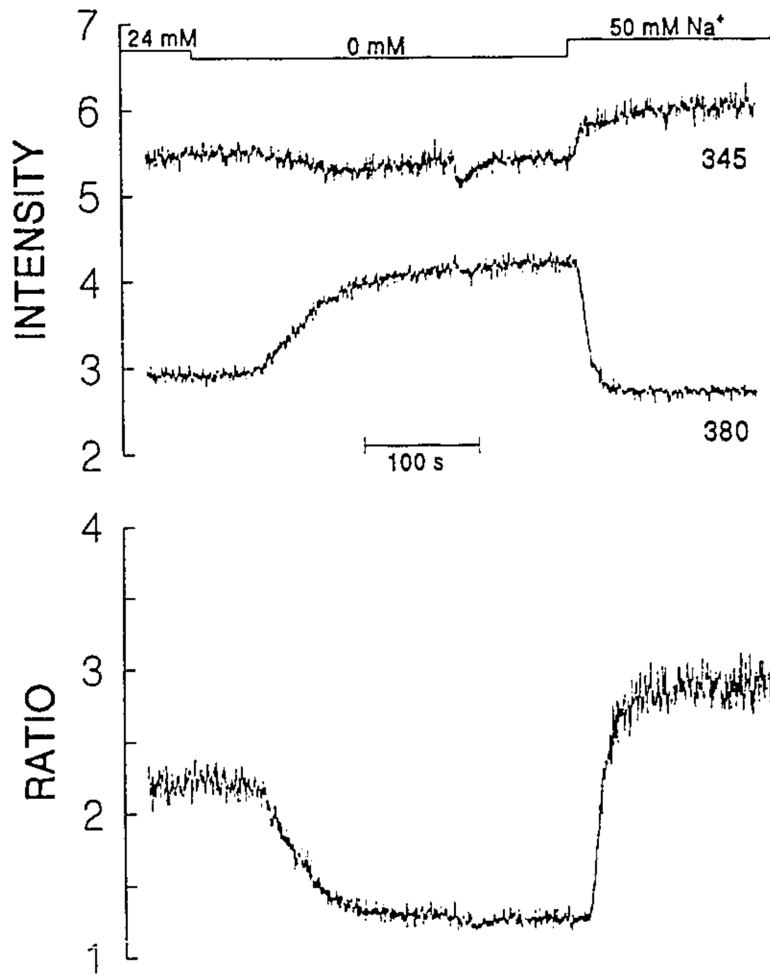


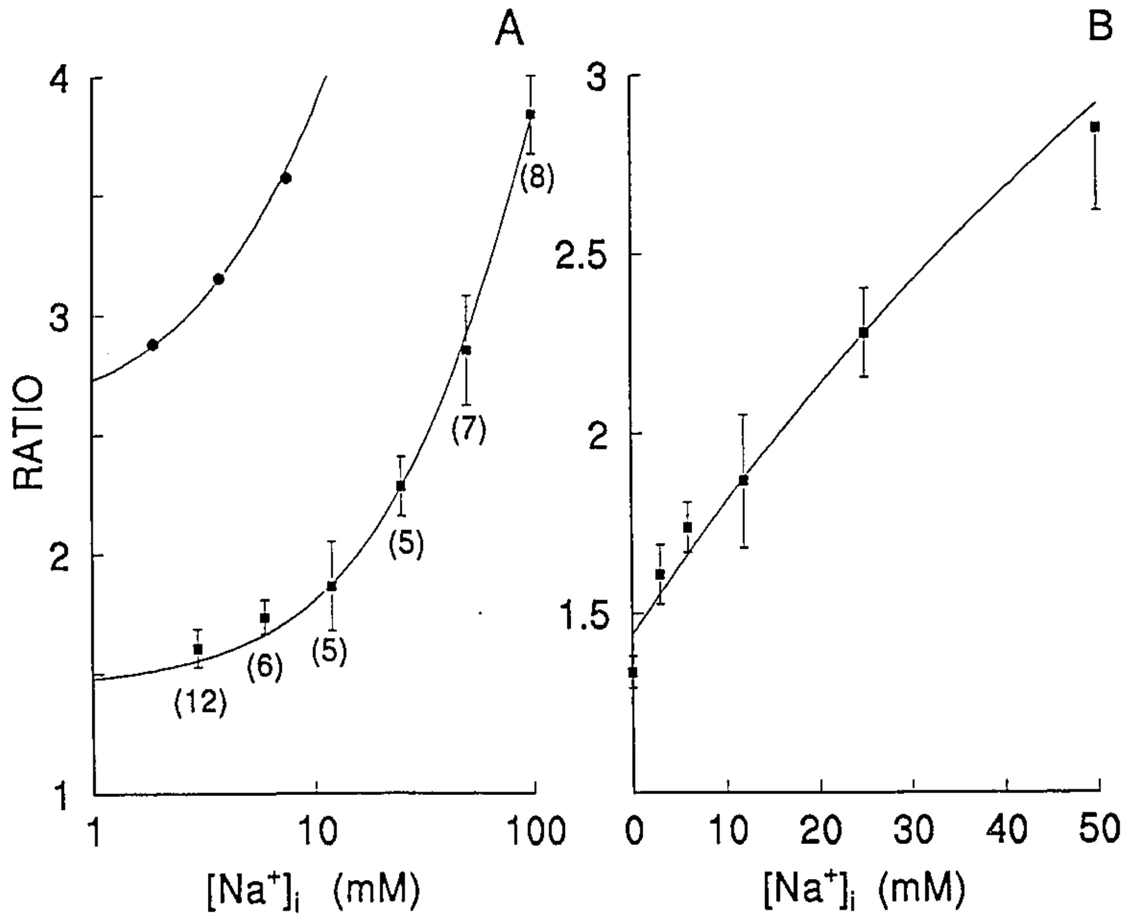
FIGURE 5. Intracellular calibration of SBFI-loaded cardiac myocytes.

Panel A: Graph of the ratios (345 over 380) as a function of Na^+ concentrations (logarithmic scale). Filled squares represent average values (± SEM) of measurements from SBFI-loaded cells maintained under the conditions described in Figure 4. The numbers in parenthesis indicate the number of cells studied at a given Na^+ concentration. The curve fitting the data has been obtained according to the calibration equation (see Methods) with the following values: C=158 mM; R_{min}=1.44; R_{100}=3.82. Filled circles are values obtained from extracellular calibration of SBFI (transposed from Figure 2).

Panel B: Graph of the ratios (345 over 380) as a function of Na^+ concentrations (as in Panel A), shown using a linear scale. This demonstrates the relative linear relationship between the ratio values and $[Na^+]$ between 0 and 50 mM.

Note: As Panel B is plotted on a linear scale, it shows the ratio value at zero mM Na^+, while the first value of Panel A is at 3mM.

FIGURE 5



also be observed from Figure 5. In other words, the intracellular environment appeared to cause a right shift of the SBFI calibration curve together with a substantial decrease of the minimum ratio. The curves shown in panel A were plotted with a logarithmic scale on the $[Na^+]$ axis. To better assess the sensitivity of the method, data are also given on a linear scale (panel B). As shown, for Na^+ concentrations between 3 and 50 mM, the increase of ratio appears to be approximately linear; with an increment of about 0.1 unit per 2.8 mM Na^+.

Overall, the results demonstrate the importance of intracellular calibrations for the determination of Na^+ concentrations in SBFI-loaded cardiac myocytes. Applying the results of this calibration to the cells studied subsequently, showed that the average $[Na^+]_i$ in steady-state conditions varied depending upon the Ca^{2+} concentration in the superfusion solution. When the concentration was 2 mM, the $[Na^+]_i$ was found to be 6.4±1.8 mM, as determined from the ratio of 1.67±0.06 (n=10). At a concentration of 0.8 mM, the $[Na^+]_i$ was 13.0±1.4 mM, as determined from a ratio of 1.89±0.043 (n=60).

EFFECT OF ISOPROTERENOL UNDER STEADY-STATE CONDITIONS

An example of the effects of isoproterenol applied under steady-state conditions in the presence of 2 mM Ca^{2+} is illustrated in Figure 6. As shown, the β-agonist failed to induce a detectable change of both $[Na^+]_i$ and transmembrane current. For ten cells investigated under these conditions, the average $[Na^+]_i$ values as measured before ISO exposure, and at 30 and 60 sec. after its introduction, were; 6.4±1.8, 6.6±1.9 and 6.6±1.8 mM respectively. Although these measurements were obtained from cells clamped at -80 mV, isoproterenol also failed to cause a significant change of $[Na^+]_i$ in 3 cells clamped at -10 mV (data not shown).

EFFECT OF ISOPROTERENOL AND GRAMICIDIN IN THE PRESENCE OF EXTERNAL Ca^{2+}

The ionophore gramicidin was used to induce an augmentation of $[Na^+]_i$ through an increase of membrane permeability to Na^+ ions. A typical example of the effect of 10 nM of gramicidin is shown in Figure 7. Exposure of the myocyte to the ionophore promptly resulted in a reversible increase of the $[Na^+]_i$ together with the gradual development of an continuously declining negative transmembrane current.

Note the relatively large current fluctuations as the $[Na^+]_i$ increases. From visual inspection, these oscillations could be clearly related to the occurrence of spontaneous cell contractions and, hence, are likely due to fluctuations of intracellular Ca^{2+}. These

FIGURE 6. Typical example of the effect of isoproterenol on a myocyte maintained under steady-state conditions.

Tracings represent simultaneous recordings of $[Na^+]_i$ (upper trace) and transmembrane current, I_m (lower trace), from a SBFI-loaded myocyte. Holding potential = -80 mV. Isoproterenol (0.5 μM) was present during the time indicated by the horizontal bar. Throughout the recording period, 20 μM of EDTA and 0.1 μM of glutathione were present. Transmembrane current data were not digitally filtered.

FIGURE 6

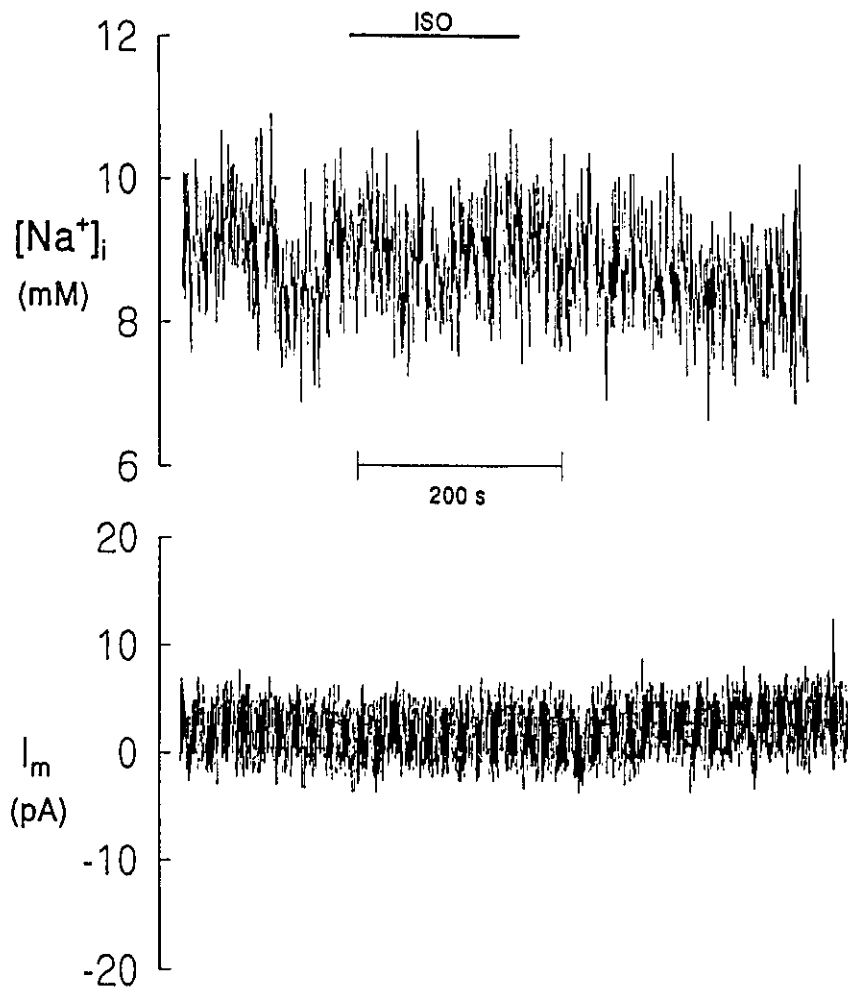
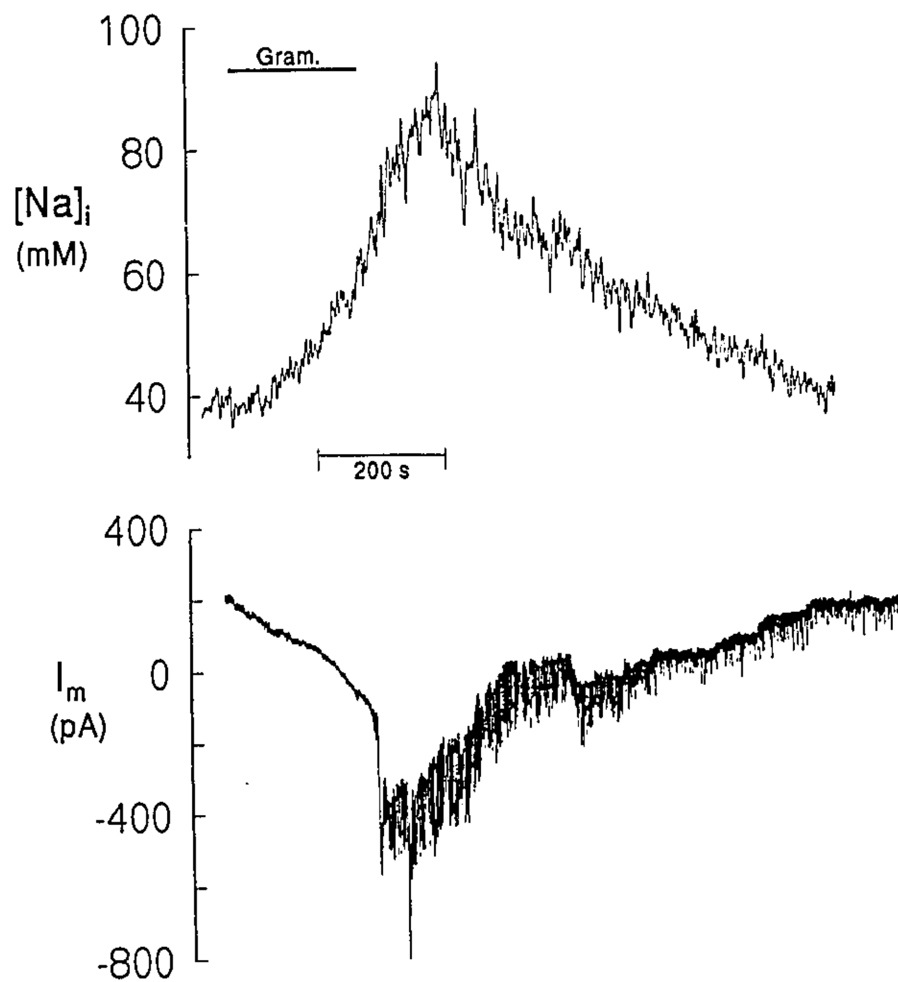


FIGURE 7. Typical example of the effect of gramicidin.

Tracings represent simultaneous recordings of $[Na^+]_i$ (upper trace) and transmembrane current, I_m (lower trace), from a SBFI-loaded myocyte. Holding potential = -70 mV. Gramicidin (10 nM) was present during the time indicated by the horizontal bar. Transmembrane current data were not digitally filtered.

FIGURE 7



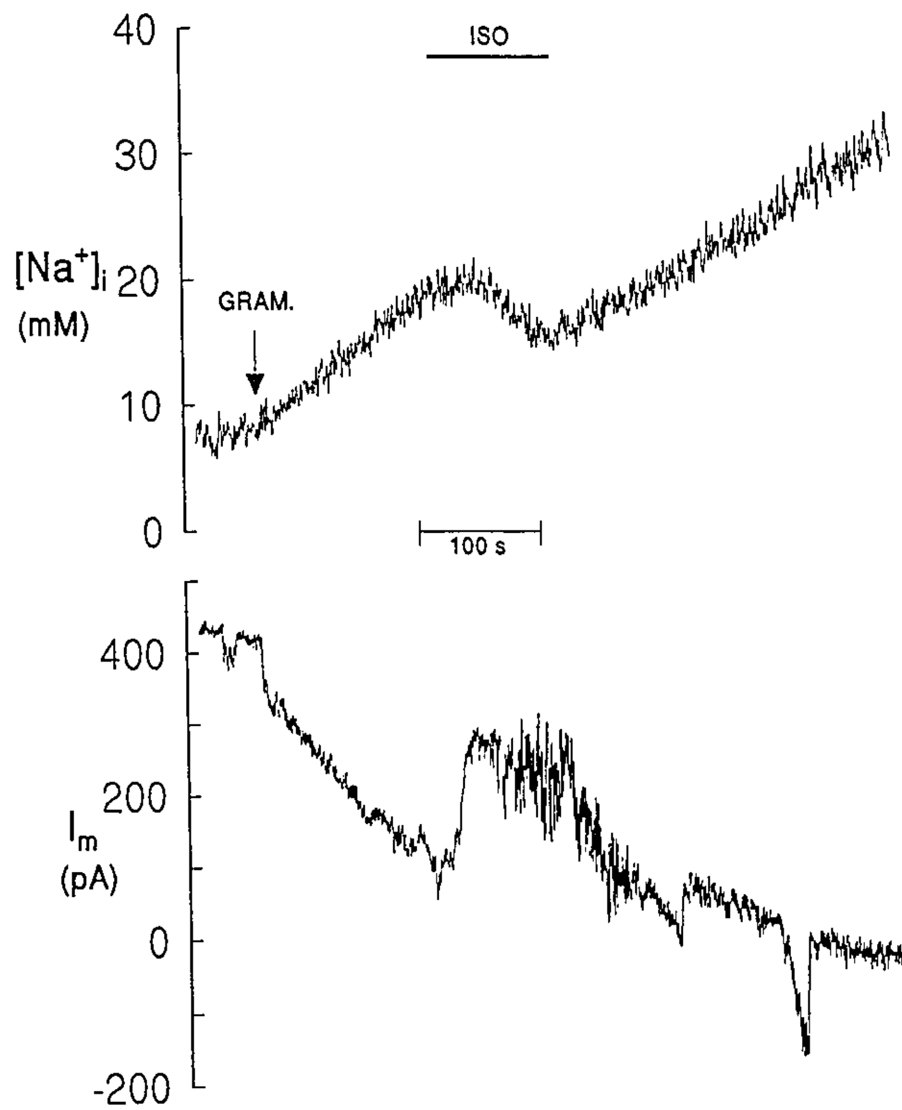
oscillations and contractions could be prevented, or at least attenuated, by the addition of 0.1 μM ryanodine in the gramicidin containing solutions. For that reason, ryanodine was always present in later experiments.

The myocytes proved to be highly susceptible to gramicidin in the presence of extracellular Ca^{2+}. Thus, preliminary experiments demonstrated that most of the myocytes rapidly underwent contracture when exposed to concentrations of gramicidin larger than 30 nM. By contrast, gramicidin concentrations less than 5 nM usually failed to demonstrate any effect on $[Na^+]_i$. In concentrations between 5 and 30 nM, the fact that the negative shift in the current was continually increasing in value indicated that the gramicidin incorporated in the membrane, and that in the extracellular solution, did not reach the state of equilibrium required to achieve a new elevated steady-state of $[Na^+]_i$. Although equilibrium might have been achieved given a longer time period, by then the $[Na^+]_i$ would have reached an undesirably high level. Furthermore, the onset of the gramicidin-induced $[Na^+]_i$ increase varied between cells. In any case, at a gramicidin concentration of 10 nM, the average rate of rise of $[Na^+]_i$, measured about three min after the $[Na^+]_i$ started to increase, was 7.3±0.9 mM/min (n=40). Of these 40 cells which were exposed to this concentration of gramicidin, only 23 survived to be further tested for the effects of isoproterenol. From this point on this Na^+ leak created by gramicidin will be referred to as the gramicidin-induced Na^+ influx.

A typical example of the effect of isoproterenol on the gramicidin-induced Na^+ influx is shown in Figure 8. The addition of isoproterenol during the rise of $[Na^+]_i$ had

FIGURE 8. Typical example of the effect of isoproterenol in the presence of gramicidin. Tracings represent simultaneous changes of $[Na^+]_i$ (upper trace) and transmembrane current, I_m (lower trace), recorded from a SBFI-loaded myocyte. Holding potential = -70 mV. The cell was first exposed to 10 nM gramicidin (indicated by the arrow). Gramicidin was present until the end of the recording. Isoproterenol (0.5 μM) was added during the time indicated by the horizontal bar.

FIGURE 8



profound effects, as the β-agonist not only reduced the gramicidin-induced Na^+ increase, but actually reversed it. Furthermore, the addition of isoproterenol caused a clear outward shift of the current required to hold the cell at the desired membrane potential. Conversely, washout of isoproterenol rapidly brought the gramicidin-induced Na^+ influx and transmembrane current back to their initial directions. It should also be mentioned that gramicidin and ryanodine were added to the superfusing solution simultaneously, and were both present throughout the experimental protocol. The presence of ryanodine seemed to prevent the previously observed oscillations in transmembrane current during exposure of the myocyte to gramicidin. Note, however, that the current oscillations did occur during the addition of isoproterenol.

Figure 9 illustrates the overall effects of isoproterenol on the gramicidin-induced Na^+ influx and holding current. On average, the amine caused a significant decrease ($p<0.05$) of Na^+ influx from 4.6±0.9 to -2.7±1.0 mM/min (n=12; see Legend for characteristics of cells represented). As can be seen with the example shown in Figure 8, this effect was entirely reversible, such that the gramicidin-induced Na^+ influx became equal, on average, to 5.8±1.5 mM/min (n=12) when measured 3 min after washout of isoproterenol (Figure 9, upper panel). Similarly, the average holding current became significantly more positive in the presence of isoproterenol (-73±86 pA), than in its absence, where values of -149±88 and -153±78 pA (n=14), before the addition of ISO, and after ISO washout respectively were observed (lower panel; see Legend for characteristics of cells represented). It should be mentioned that the relatively large

FIGURE 9. Effect of isoproterenol on gramicidin-induced Na^+ influx and transmembrane current.

Upper panel: rate of change of $[Na^+]_i$ as measured 30 sec before exposure to isoproterenol (CONTROL), 90 sec after the addition of isoproterenol (ISO) and 180 sec after its removal (WASHOUT).

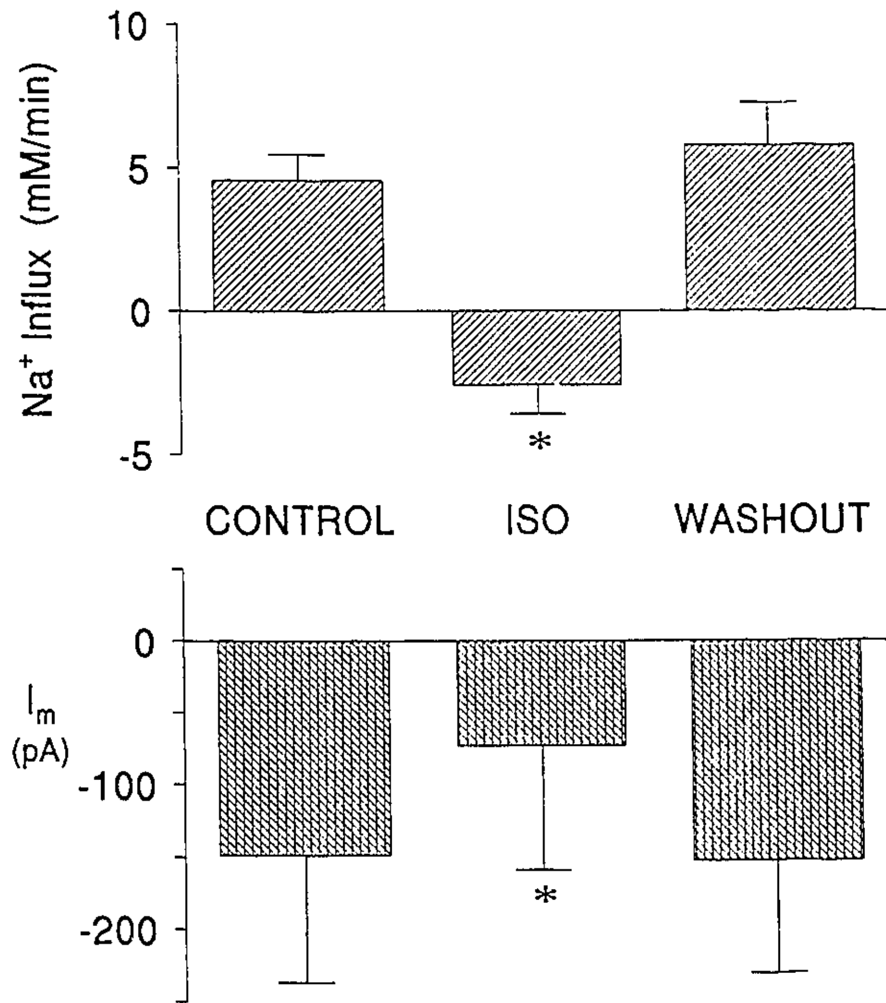
Data are average values ± SEM, obtained from only those 12 cells from which measurements of fluorescent data were possible throughout the entire experimental protocol; CONTROL, ISO and WASHOUT.

Lower panel: transmembrane current values as measured immediately before (CONTROL), 30 sec after the addition of isoproterenol (ISO) and 150 sec after its removal (WASHOUT). Holding potential was equal to -70 mV.

Data are average values ± SEM, obtained from only those 14 cells from which measurements of transmembrane current were possible throughout the entire experimental protocol; CONTROL, ISO and WASHOUT. Note that the population of cells represented in the lower panel is not the same as that of the upper panel.

* Significantly different from their respective CONTROL and WASHOUT values, as determined from paired T-tests.

FIGURE 9



standard errors associated with the current measurements were due to the great variability of the effect of gramicidin on the holding current. However, a clear positive shift was observed in 10 of the 14 cells reported, thereby giving a highly significant difference ($p<0.007$) when statistically compared by paired t-test.

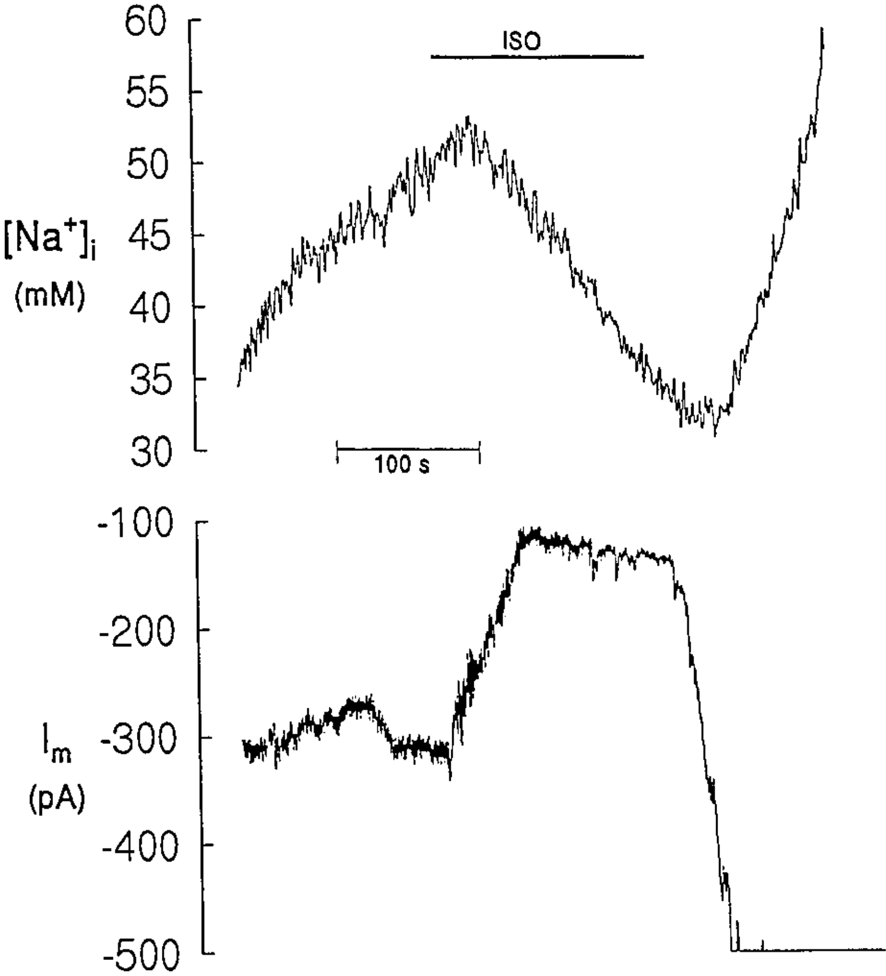
EFFECT OF ISOPROTERENOL AND GRAMICIDIN IN THE ABSENCE OF EXTERNAL Ca^{2+}

In the first series of experiments, the myocytes were incubated in normal, Ca^{2+}-containing, Tyrode's solution. In this set of experiments, the extracellular Ca^{2+} was removed (replaced with 0.5 mM EGTA) when the cells were put in the superfusion chamber, i.e. just before their actual impalement and SBFI loading. At the time of impalement the average membrane potential for this group of cells was found to be -58.9±2.8 mV (n=23). The average steady-state $[Na^+]_i$ measured under these conditions was found to be equal to 11.9±3.9 mM (n=23), a value not significantly different than the 13.0±1.4 mM measured in the presence of extracellular Ca^{2+}. Addition of gramicidin also induced an increase of $[Na^+]_i$, although the effect appeared to be smaller than with cells exposed to external Ca^{2+}. Thus, despite the fact that the gramicidin concentration used here was 30 nM, the average rate of rise measured 3 min after the $[Na^+]_i$ started to increase was equal to 2.13±0.7 mM/min (n=24), a value significantly smaller ($p<0.006$) than that measured in myocytes maintained in the presence of external Ca^{2+} (7.3±0.9 mM/min) (n=40). Note that the 24 cells represent the total number injected with SBFI and studied under 0 mM Ca^{2+} and 0.5 mM EGTA throughout this research project.

A typical example of the effect of isoproterenol recorded under these conditions is illustrated in Figure 10. It can be seen that ISO affected both the $[Na^+]_i$ and the holding current in a manner similar to that observed in the presence of external Ca^{2+}; i.e. reversal

FIGURE 10. Typical example of the effect of isoproterenol in the absence of extracellular Ca^{2+}. Tracings represent simultaneous changes of $[Na^+]_i$ (upper trace) and transmembrane current, I_m (lower trace), recorded from a SBFI-loaded myocyte. Holding potential = -70 mV. Prior to SBFI injection, the superfusion solution was switched from the normal Tyrode's solution to a Ca^{2+}-free solution containing 0.5 mM EGTA. About 5 min after SBFI loading, the cell was exposed to 30 nM gramicidin, followed 8 min later by the addition of 0.5 μM isoproterenol which was present during the time indicated by the horizontal bar. Note that the I_m recording went out of scale following ISO washout.

FIGURE 10



by ISO of the gramicidin-induced Na^+ influx (upper panel), together with an outward shift of the holding current (lower panel). These effects were promptly reversed upon washout of the β-adrenergic agonist. It should be mentioned that despite the removal of Ca^{2+} from the extracellular solution, some cells (40%) still exhibited some contractile activity when exposed to ISO, which indicated that their intracellular store of Ca^{2+} in the sarcoplasmic reticulum was not completely depleted.

Figure 11 describes the average values of the $[Na^+]_i$ changes (upper panel), and the average I_m (lower panel), measured under these conditions (see Legend for characteristics of cells represented). Thus, the rate of $[Na^+]_i$ rise significantly decreased ($p<0.016$), from 1.1±0.9 to -6.2±2.0 mM/min during isoproterenol exposure, while the holding current increased from -44±62 to 15±61 pA ($p<0.025$) during the same period. These changes induced by isoproterenol were completely reversible such that, approximately three minutes after wash out of the ISO, the gramicidin-induced Na^+ influx became equal to 3.8±1.9 mM/min while the holding current shifted inwardly to -90±87 pA. As previously mentioned regarding Figure 9, the relatively large standard errors associated with the current measurements were due to the great variability of the effect of gramicidin on the holding current. However, a clear positive shift was observed in 8 of the 10 cells reported in this case, giving a significant difference when statistically compared by paired t-test.

FIGURE 11. Effect of isoproterenol on gramicidin-induced Na^+ influx and transmembrane current in the absence of extracellular Ca^{2+}.

As described in the legend of Figure 10, measurements were performed on cells superfused with Ca^{2+}-free solutions containing 0.5 mM EGTA.

Upper panel: rate of change of $[Na^+]_i$ as measured 30 sec before exposure to isoproterenol (CONTROL), 90 sec after the addition of isoproterenol (ISO) and 180 sec after its removal (WASHOUT).

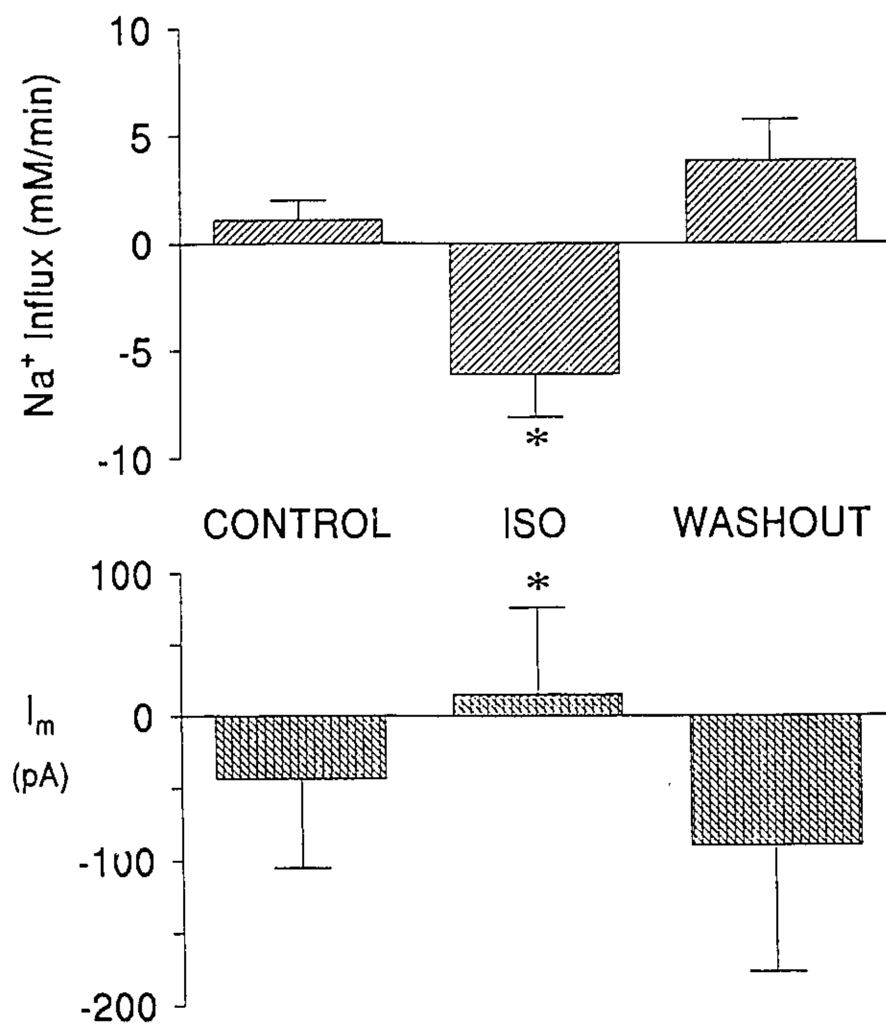
Data are average values ± SEM, obtained from only those 10 cells from which measurements of fluorescent data were possible throughout the entire experimental protocol; CONTROL, ISO and WASHOUT.

Lower panel: transmembrane current values as measured immediately before (CONTROL), 30 sec after the addition of isoproterenol (ISO) and 150 sec after its removal (WASHOUT). Holding potential was equal to -70 mV throughout.

Data are average values ± SEM, obtained from only those 10 cells from which measurements of transmembrane current were possible throughout the entire experimental protocol; CONTROL, ISO and WASHOUT. Note that the population of cells represented in the lower panel is not the same as that of the upper panel.

* Significantly different from their respective CONTROL and WASHOUT values, as determined from paired T-tests.

FIGURE 11



EFFECT OF ISOPROTERENOL IN THE PRESENCE OF STROPHANTHIDIN

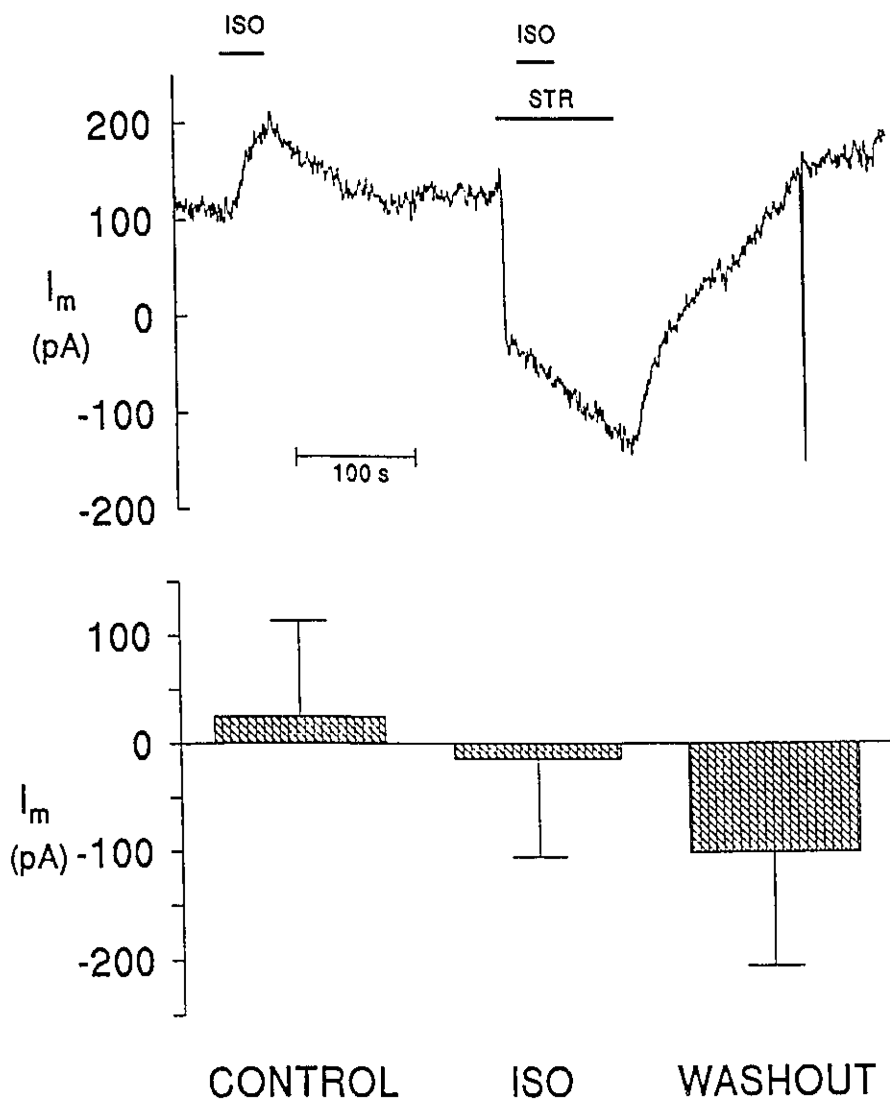
In order to test whether the effects of isoproterenol were due to a stimulation of the Na-K pump, experiments were performed in the presence of strophanthidin, a Na-K pump inhibitor. Unfortunately, in Ca^{2+} containing solutions, the myocytes could not withstand the simultaneous exposure to gramicidin, strophanthidin and isoproterenol for a long period. Consequently, the recording of $[Na^+]_i$ changes was difficult because it required long exposures to the three agents. On the other hand, the changes of transmembrane currents in response to isoproterenol occur more quickly than those of $[Na^+]_i$ (see Figure 8) and, hence, do not require such long exposures. Accordingly, a series of experiments involving only transmembrane current recordings and brief applications of strophanthidin and isoproterenol were carried out. A typical recording is given in the upper panel of Figure 12, with the average values presented in the lower panel. As shown, a short exposure to ISO still caused the expected outward shift in the presence of gramicidin. On the other hand, addition of 100 μM strophanthidin resulted in a rapid drop of the holding current. On average, the current shifted from 141±65 to 40±90 pA (as measured 15 sec after strophanthidin addition, n=10). As Figure 12 illustrates, this rapid drop was followed by a slow but continuous shift of the current to greater negative values. Under these conditions, exposure of the myocyte to isoproterenol failed to induce any detectable outward current. This apparent lack of effect of isoproterenol in the presence of strophanthidin was found in all of the ten cells studied under these conditions. Although

FIGURE 12. Effect of isoproterenol on transmembrane current in the presence of strophanthidin and Ca^{2+}.

Upper panel: recording of transmembrane current from a cell maintained at -70 mV. Gramicidin, added about 3 min prior to the beginning of the recording, was present throughout at a concentration of 10 nM. Cell exposures to 0.5 μM isoproterenol (ISO) and 100 μM strophanthidin (STR) are indicated by the horizontal bars.

Lower panel: average values of transmembrane current measured immediately before (CONTROL), 30 sec after the addition of isoproterenol (ISO) and 30 sec after its removal (WASHOUT), as recorded in the presence of 100 μM strophanthidin. Data were obtained from 10 cells.

FIGURE 12



an effect of ISO could have been partially masked by the slow declining current observed in the presence of strophanthidin, this observation strongly indicates that the isoproterenol-induced outward current is related to the Na-K pump.

Contrary to the experiments that were performed on cells bathed in solutions containing Ca^{2+}, those carried out in the absence of Ca^{2+} allowed the prolonged exposure to strophanthidin in the presence of gramicidin and, hence, the measurement of the $[Na^+]_i$ changes. Such an experiment is illustrated in Figure 13. As expected, the addition of strophanthidin initiated a rapid increase of $[Na^+]_i$ (upper panel). On average, the rate of $[Na^+]_i$ rise, measured 2.5 min after the addition of 100 μM strophanthidin, was equal to 6.5±1.7 mM/min (n=13), a value significantly larger ($p<0.001$) than the 2.1±0.7 mM/min observed in the same cells with gramicidin alone, before the addition of the Na-K pump blocker. As previously described for the measurements made in the presence of extracellular Ca^{2+}, strophanthidin also caused an inward shift of the holding current (lower panel). The average current dropped from 40±54 to -64±91 pA (n=8) after 15 sec exposure to strophanthidin. This inward shift of 104±42 pA is similar to the 100±32 pA measured from cells maintained in Ca^{2+} (figure 12).

Figure 13 also demonstrates that isoproterenol, when applied during strophanthidin exposure, completely failed to affect the rate of rise of the $[Na^+]_i$ as well as the holding current. This inhibition of the ISO effects was observed in all tested cells, as depicted in Figure 14, which shows that the rate of rise measured after washout of ISO was significantly smaller ($p<0.027$) than that measured during exposure to the β-agonist;

FIGURE 13. Typical example of the effects of isoproterenol in the presence of strophanthidin and EGTA.

Tracings represent simultaneous changes of $[Na^+]_i$ (upper trace) and transmembrane current, I_m (lower trace), recorded from an SBFI-loaded myocyte. Holding potential = -70 mV. The cell was first exposed to 30 nM gramicidin and zero Ca^{2+} (0.5 mM EGTA), followed 3 min later by the addition of 100 µM strophanthidin (indicated by the arrow). Gramicidin, EGTA and strophanthidin were present until the end of the recording. The cell was further exposed to isoproterenol (0.5 µM) during the time indicated by the horizontal bar. Note that the I_m recording went out of scale shortly after ISO washout.

FIGURE 13

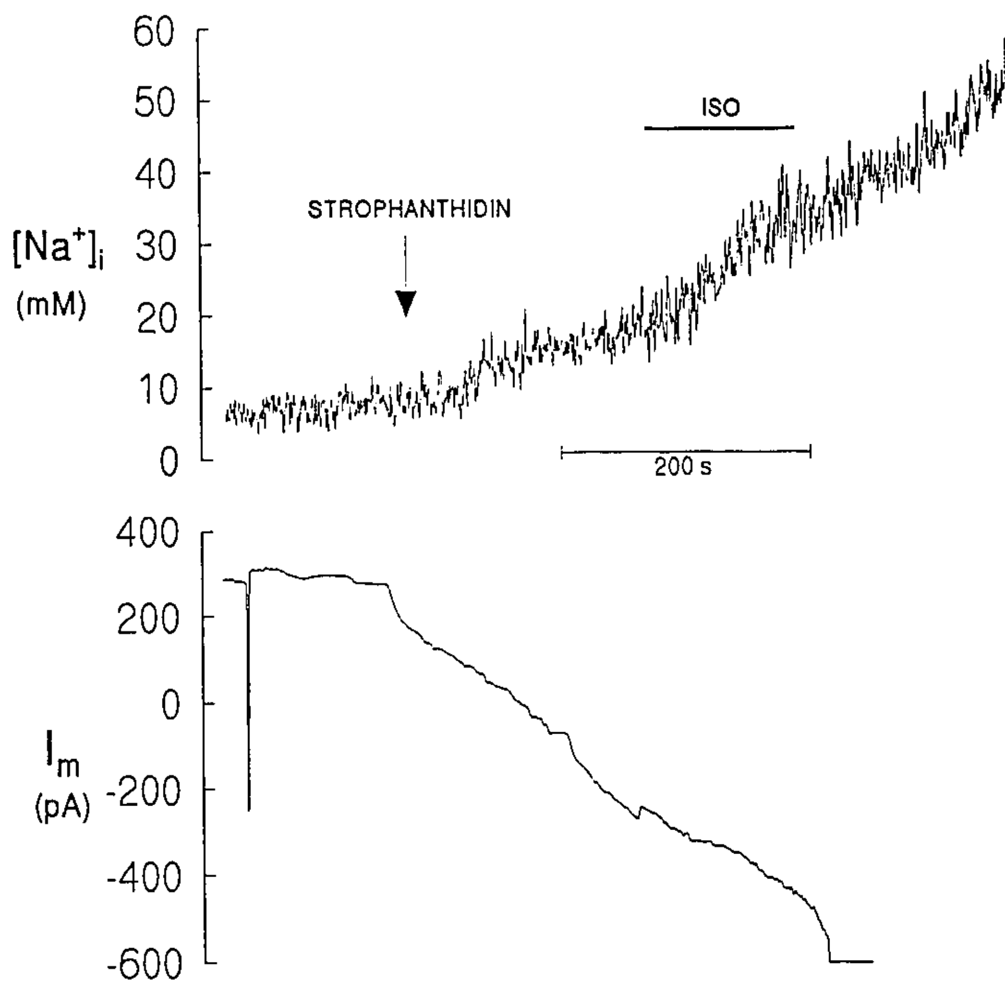


FIGURE 14. Effect of isoproterenol on gramicidin-induced Na^+ influx and transmembrane current in the presence of strophanthidin and EGTA.

As described in the legend of Figure 13, measurements were performed on cells superfused with Ca^{2+}-free solutions containing 0.5 mM EGTA, 10 nM gramicidin and 100 μM strophanthidin.

Upper panel: rate of change of $[Na^+]_i$ as measured 30 sec before exposure to isoproterenol (CONTROL), 90 sec after the addition of isoproterenol (ISO) and 180 sec after its removal (WASHOUT).

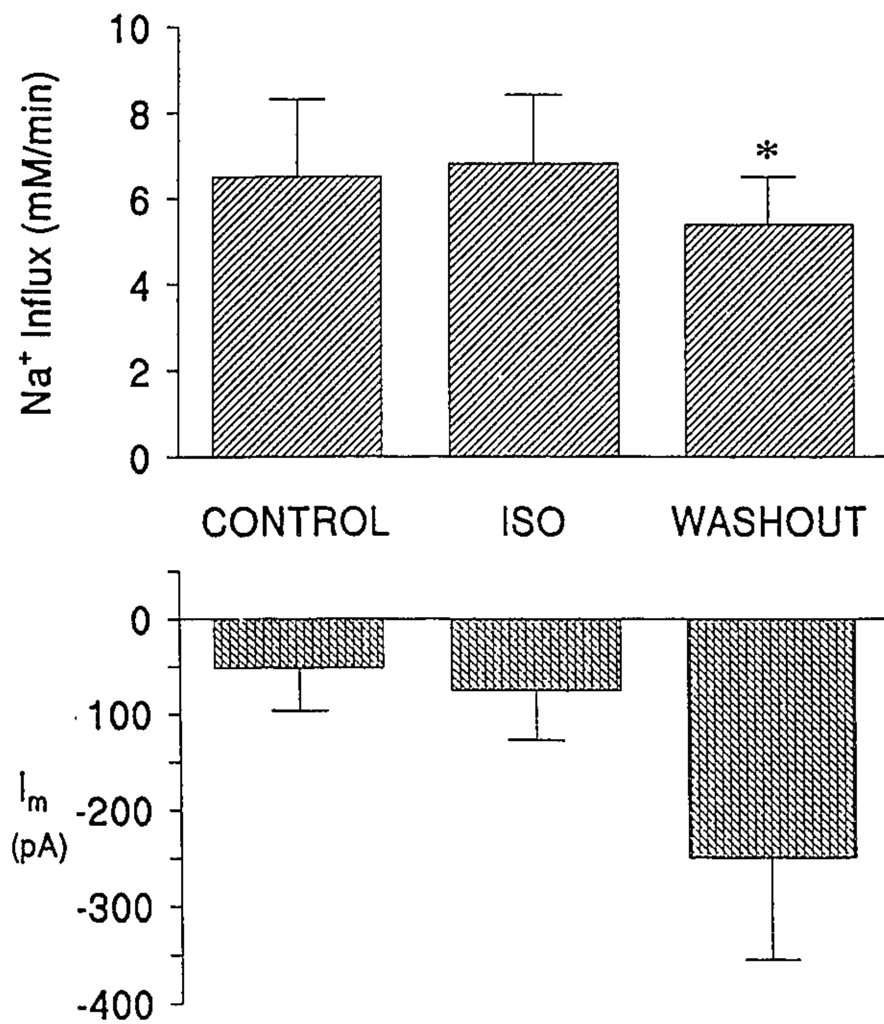
Data are average values ± SEM, obtained from only those 12 cells from which measurements of fluorescent data were possible throughout the entire experimental protocol; CONTROL, ISO and WASHOUT.

Lower panel: transmembrane current values as measured immediately before (CONTROL), 30 sec after the addition of isoproterenol (ISO) and 150 sec after its removal (WASHOUT). Holding potential was equal to -70 mV throughout.

Data are average values ± SEM, obtained from only those 6 cells from which measurements of transmembrane current were possible throughout the entire experimental protocol; CONTROL, ISO and WASHOUT. Note that the population of cells represented in the lower panel is not the same as that of the upper panel.

* Significantly different from their respective CONTROL and ISO values, as determined from paired T-tests.

FIGURE 14



an effect opposite to that observed in gramicidin alone (see Figures 9 and 11). This apparent discrepancy could be related to the level of intracellular Na^+ reached at that time. Thus the initial $[Na^+]_i$ values increased, on average, from 28.4±6.0 mM (before ISO), to 40.8±8.4 mM (during ISO exposure), and to 56.2±13.2 mM (after ISO wash out). By comparison the equivalent values, in the absence of strophanthidin, were; 29.7±5.6, 32.9±6.7 and 26.0±6.1 mM respectively. The inhibition by strophanthidin of ISO effects is also apparent from the holding current data (Figure 14, lower panel), where the I_m continued to decline during ISO exposure. This behaviour is similar to that reported for the measurements performed with short exposures to ISO in the presence of external Ca^{2+} (Figure 12).

EFFECT OF ISOPROTERENOL ON BAPTA-LOADED MYOCYTES

In order to ensure complete depletion of intracellular Ca^{2+}, myocytes were incubated for 2 to 3 hours before SBFI loading in Ca^{2+}-free Tyrode's solution containing 0.5 mM EGTA and 25 μM BAPTA-AM (see Methods Section). It should be noted, however, that upon exposure to isoproterenol, contractile activity was observed in 3 of the 19 cells tested for the effects of the β-agonist, an indication that intracellular Ca^{2+} was not completely chelated by BAPTA. These 3 cells were therefore excluded from the analysis.

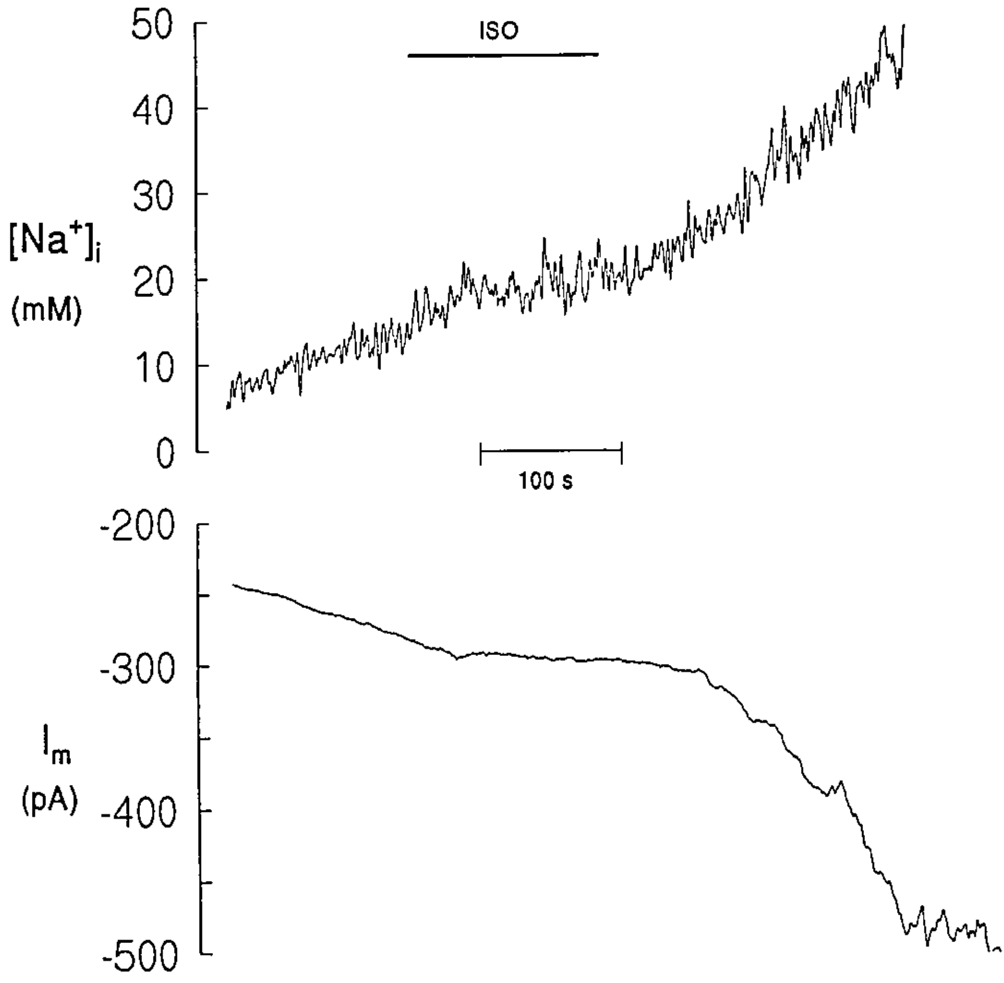
The BAPTA-AM preincubated cells, which were kept in zero Ca^{2+}, were often depolarised, the average resting membrane potential being equal to -45.8±2.8 mV. Due to this depolarised state, the myocytes were subsequently clamped at either -70 mV or -10 mV, depending on the initial resting potential and the membrane current needed to hold the potential at these levels. Basal $[Na^+]_i$ found in the cells clamped at -70 mV was 21.5±9.2 mM (n=9). Although this value appears higher than comparable values observed in the presence of either Ca^{2+} or EGTA, statistical tests failed to detect any significant difference. Basal $[Na^+]_i$ measured under -10 mV voltage-clamping was equal to 30.8±8.8 mM (n=8). This value is significantly higher ($p<0.03$ for both; Mann-Whitney *U* test) than the 13.0±1.4 and 11.9±3.9 mM, measured from cells maintained in Ca^{2+} or EGTA containing Tyrode's solution respectively.

Figure 15 illustrates a typical example of the effect of ISO on the gramicidin-induced Na^+ leak from myocytes preincubated in BAPTA. In this case, exposure to ISO

FIGURE 15. Typical example of the effect of isoproterenol on a myocyte preincubated in BAPTA-AM.

Tracings represent simultaneous changes of $[Na^+]_i$ (upper trace) and transmembrane current, I_m (lower trace), recorded from a SBFI-loaded myocyte. Holding potential = -70 mV. The cell was preincubated for about 3 hours in a Ca^{2+}-free solution containing 0.5 mM EGTA and 25 μM BAPTA-AM. About 5 min after SBFI loading, the cell was exposed to 30 nM gramicidin, followed 3 min later by the addition of 0.5 μM isoproterenol which was present during the time indicated by the horizontal bar. Gramicidin and EGTA were present throughout.

FIGURE 15



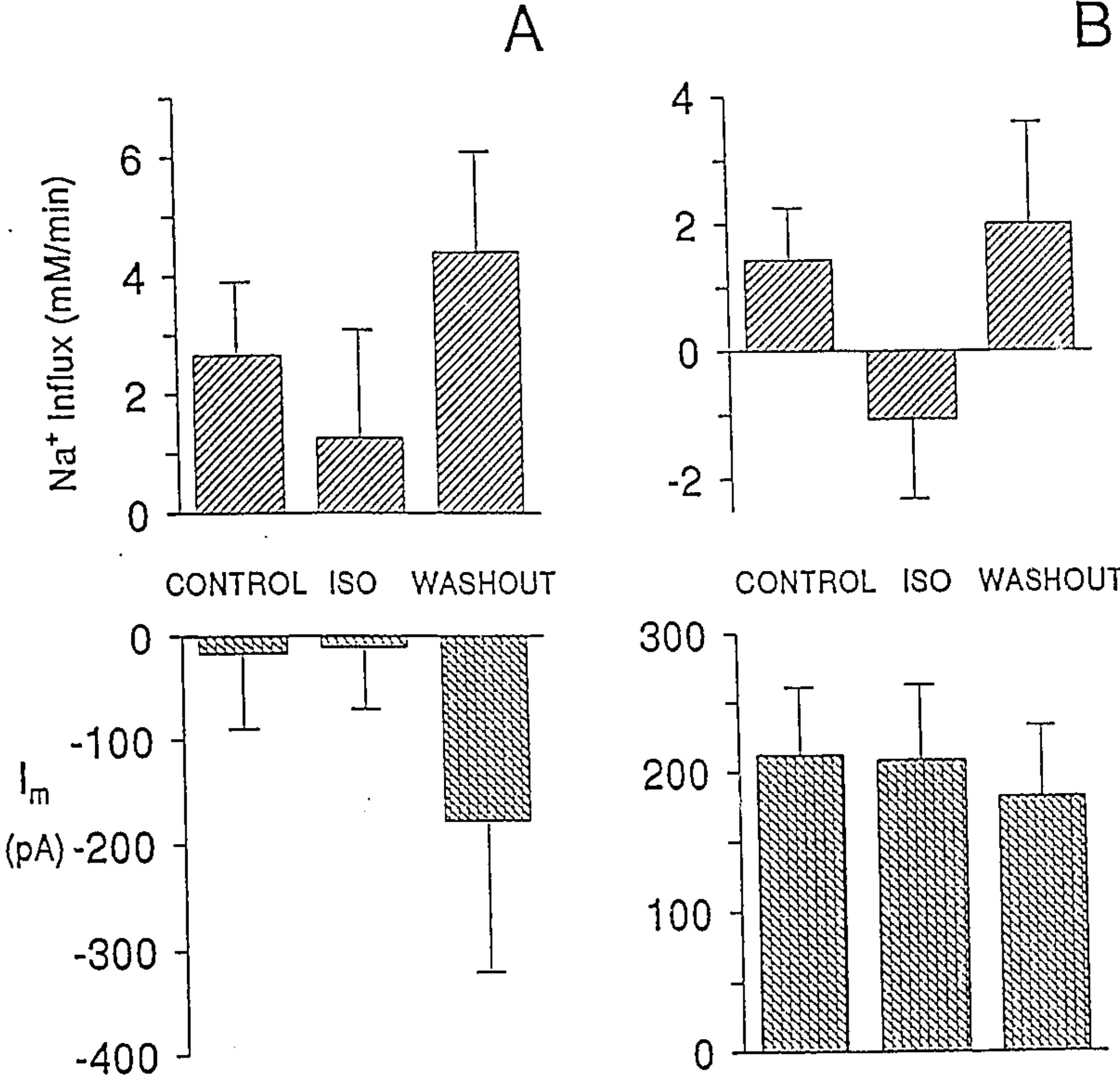
caused only a small change in the rate of $[Na^+]_i$ rise, slowing the rise rather than reversing it (Figures 8 and 10, upper panels). Similarly, ISO appeared to affect the holding current by reducing the inward shift that progressively developed during the gramicidin exposure, but this effect is far less evident than the positive shift described previously (Figures 8 and 10, lower panels). In the example illustrated in Figure 15, the cell was clamped at -70 mV, but similar results were obtained for cells maintained at -10 mV. The average values measured at these two membrane potentials are presented in Figure 16. Several observations can be made from this figure. The gramicidin-induced Na^+ influx measured at -10 mV (1.4±0.8 mM/min) (n=7) and -70 mV (2.7±1.2 mM/min) (n=7) are significantly smaller ($p<0.002$ and 0.007 respectively, Mann-Whitney *U* test) than that measured under control conditions (7.3±0.9 mM/min) (n=40). Note that the average rate of the leak at -70 mV is almost twice that at -10 mV (Figure 16, upper panels A and B), an expected result considering the large difference between the electrical driving forces. Similarly, the holding current at -10 mV (212±49 pA) is much higher than that at -70 mV (-17±73 pA) (lower panels A and B). More importantly, this figure demonstrates that the effect of ISO on both the gramicidin-induced Na^+ influx, and the transmembrane current, appeared greatly reduced at both holding potentials. In fact, the analysis of variances did not demonstrate significant differences from their respective control and washout values. It should be noted, however, that this is likely due to the attenuated effect of ISO under these conditions, as well as the relatively low number of samples used for the comparative tests, since six of the seven cells tested under both holding potentials demonstrated a clear

FIGURE 16. Effect of isoproterenol on myocytes incubated in the presence of BAPTA-AM. For each panel, the upper graph represents the rate of change of $[Na^+]_i$ as measured 30 sec before exposure to isoproterenol (CONTROL), 90 sec after the addition of isoproterenol (ISO) and 180 sec after its removal (WASHOUT), while the lower graph represents the transmembrane current values as measured immediately before (CONTROL), 30 sec after the addition of isoproterenol (ISO) and 150 sec after its removal (WASHOUT).

Panel A: Holding potential equal to -70 mV throughout. Average values were obtained from 7 cells (upper graph) and 6 cells (lower graph).

Panel B: Holding potential equal to -10 mV throughout. Average values were obtained from 7 cells (upper graph) and 8 cells (lower graph).

FIGURE 16



decrease in the rate of Na^+ influx. In fact, combining the measurements obtained at both potentials would lead to a statistically significant effect of ISO on the rate of the rise of $[Na^+]_i$. Thus, on average, the gramicidin-induced Na^+ influx significantly ($p<0.02$) decreased from 2.0±0.7 to 0.1±1.1 mM/min in the presence of ISO, and increased to 3.2±1.2 mM/min after wash-out (n=14). Although changing the holding voltage did influence the values of the transmembrane current, the changes caused by ISO appeared not to be affected by the membrane potential. Since statistical comparisons of the changes induced by ISO showed no significant difference between the two groups, the apparent lack of effect of membrane voltage on ISO-induced shifts of both Na^+ leak and transmembrane current justifies combining the results from the two groups for comparative purposes with other experimental conditions.

OVERALL EFFECTS OF ISOPROTERENOL AND DEPENDENCY ON INTRACELLULAR Na^+

To summarize the overall effects of isoproterenol described above, Figure 17 illustrates the changes in gramicidin-induced Na^+ influx (upper panel) and transmembrane current (lower panel) that occurred upon exposure to the β-agonist. The changes reported in this figure represent the values obtained from those cells where measurements were possible under control conditions and during exposure to isoproterenol. The number of measurements for some of these groups differs slightly from the data sets previously presented because these also include the recordings that were carried out without involving the washout of ISO. In any case, none of the conclusions would be altered by these additions. The major points to be drawn from this figure are as follows:

1) Isoproterenol caused a significant decrease of gramicidin-induced Na^+ influx in all treatments studied, except in those experiments involving strophanthidin.

2) This effect of ISO (above) was not altered by the removal of extracellular Ca^{2+} at the time of impalement (ISO-induced change in Na^+ influx being equal to -6.7±1.1 and -8.0±2.5 mM/min in Ca^{2+} and EGTA, respectively).

3) The incubation of the myocytes in the absence of Ca^{2+}, and in the presence of BAPTA-AM, substantially attenuated the effect of ISO on the gramicidin-induced Na^+ influx (-2.5±0.6 mM/min).

4) ISO also caused a positive shift of the holding current in the presence or absence of

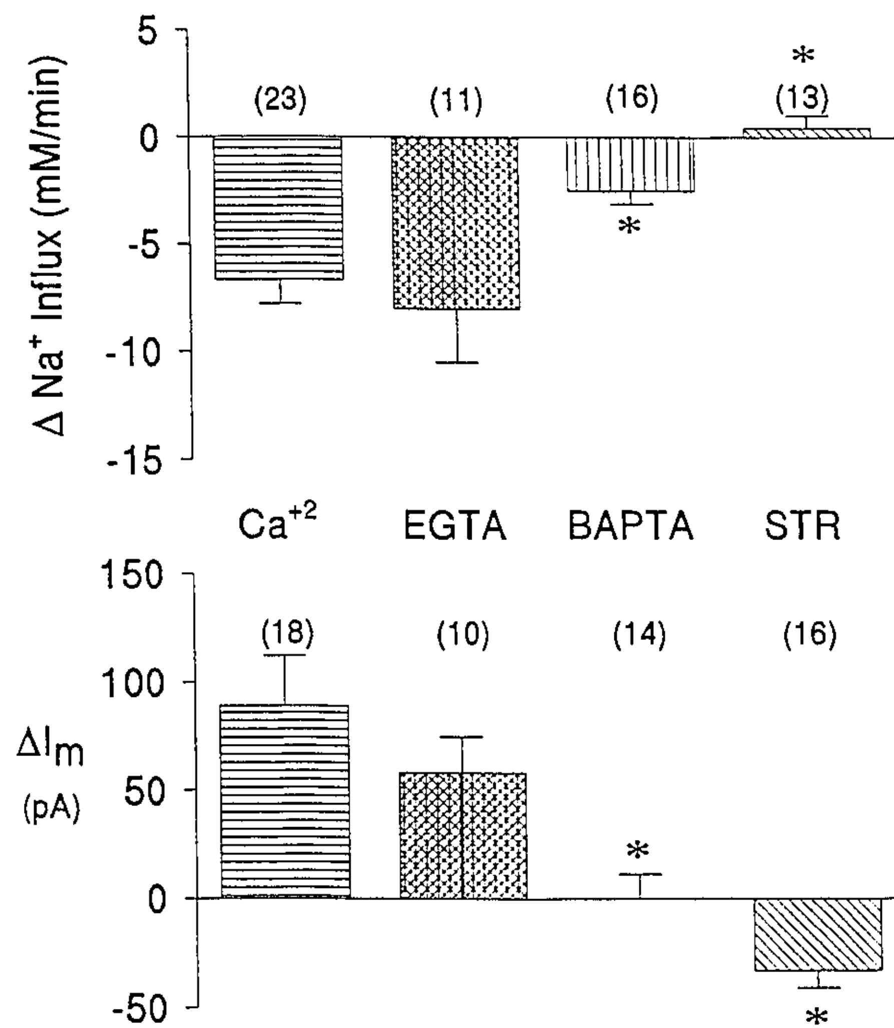
FIGURE 17. Overall effects of isoproterenol on gramicidin-induced Na^+ influx and transmembrane current.

ΔNa^+ influx (upper panel) and ΔI_m (lower panel) are the differences between the values measured in the presence of isoproterenol and those measured before its addition. The four groups labelled Ca^{2+}, EGTA, BAPTA and STR refer to the cells exposed to solutions containing Ca^{2+}, EGTA, BAPTA·AM or strophanthidin, respectively. Holding potential was -70 mV for all the conditions except for the BAPTA group, which includes cells studied at either -70 or -10 mV. Numbers in parenthesis indicate the number of cells studied for each group.

The STR group reported in the lower panel represents the combination of cells measured in the presence and absence of extracellular Ca^{2+}.

* Significantly different ($p<0.05$) from the average values in the other three groups.

FIGURE 17



extracellular Ca^{2+} ions (89±23 and 58±17 pA, respectively) although not in myocytes pre-incubated in BAPTA-AM.

5) In the presence of strophanthidin, the holding current shifted negatively even when the myocytes were exposed to ISO.

A possible explanation which could account for the significant reduction of the ISO effects after prolonged incubation in BAPTA-AM may be that the $[Na^+]_i$ levels differed between experimental conditions. However, the average $[Na^+]_i$ values reached at the time of exposure to ISO were very similar among the three groups; 33.3±8.4 mM in BAPTA-AM, 32.1±3.3 mM in Ca^{2+} and 31.9±6.2 mM in EGTA. Although the standard deviations of these values indicate a large variation, they still fall within the same range of $[Na^+]_i$.

Nevertheless, analysis of the results indicates that the magnitude of the effects of ISO is related to the $[Na^+]_i$, as demonstrated in Figure 18. Thus, for cells not preincubated in BAPTA-AM, the regression analysis gives a highly significant ($p<0.001$ and $p<0.002$ for measurements in Ca^{2+} and in EGTA, respectively) inverse relationship between the ISO-induced change in Na^+ influx and the level of intracellular Na^+ present at the time of the introduction of ISO (upper panel). Similarly, the outward shift induced by ISO is positively correlated to $[Na^+]_i$ for both the Ca^{2+} and the EGTA groups ($p<0.04$ and $p<0.001$, respectively) (lower panel). On the other hand, measurements in the cells preincubated in BAPTA-AM fail to demonstrate any significant correlation between the effects of ISO and $[Na^+]_i$.

FIGURE 18. Correlation between the effects of isoproterenol and intracellular Na^+ concentration.

Δ Na^+ influx (upper panel) and ΔI_m (lower panel) are the differences between the values measured in the presence of isoproterenol and those measured before its addition. Data are plotted as a function of intracellular Na^+ concentrations measured at the time of cell exposure to isoproterenol. Cells studied in the presence of extracellular Ca^{2+} are represented by squares, those studied in EGTA by triangles and those preincubated in BAPTA-AM by asterisks. The lines were obtained from linear fitting of these three sets of data points. The solid, dashed and dotted lines correspond to the Ca^{2+}, EGTA and BAPTA-AM groups, respectively. The lines were fitted according to the linear relationship: $y = a + bx$, where x is $[Na^+]_i$ and y is the Δ Na^+ influx or ΔI_m. The values of the constants a and b corresponding to each fitting are as follows:

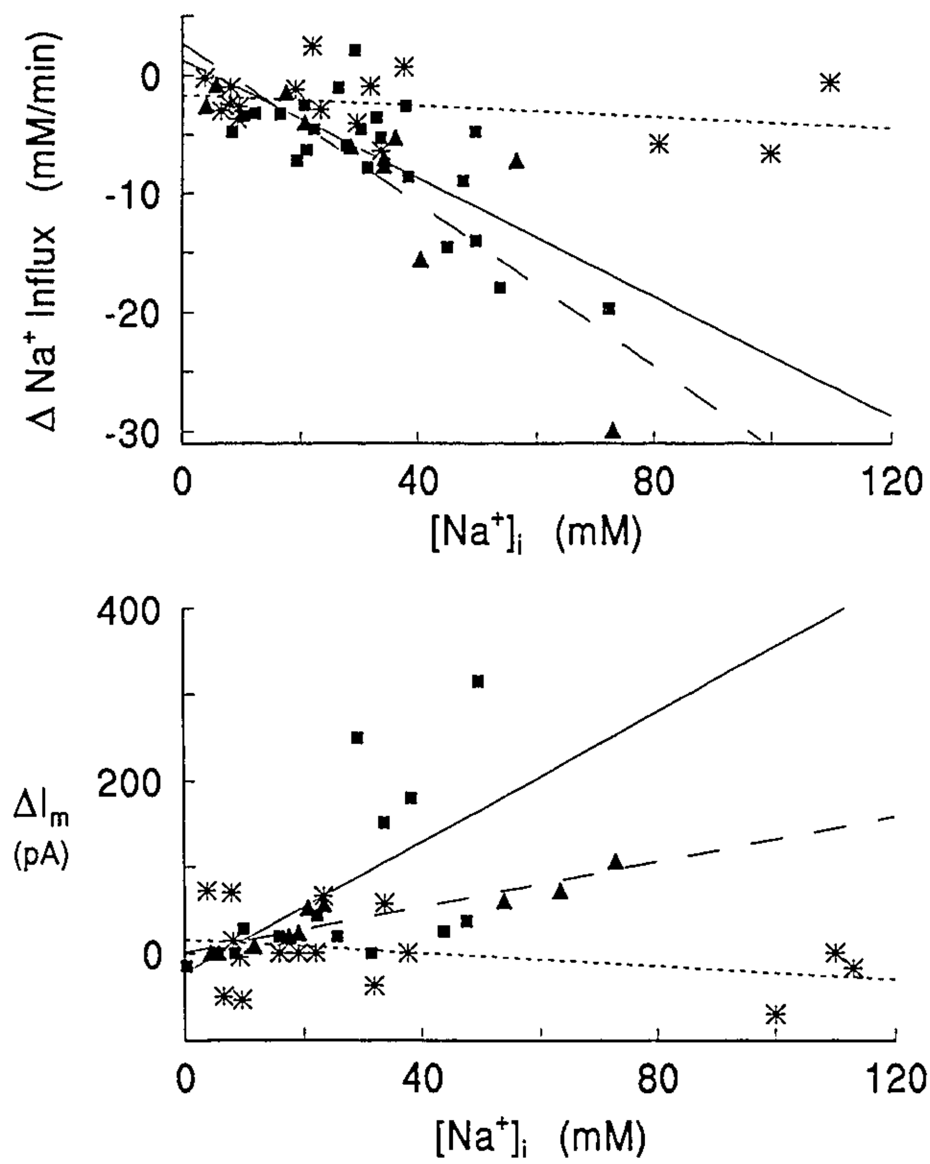
Upper panel:

	a (mM/min)	b (min^{-1})
Ca^{2+}	1.26	-0.25
EGTA	2.68	-0.34
BAPTA-AM	-1.67	-0.02

Lower panel:

	a (pA)	b (pA/mM)
Ca^{2+}	-22	3.8
EGTA	2	1.3
BAPTA-AM	16	-0.4

FIGURE 18



DISCUSSION

DETERMINATION OF INTRACELLULAR Na^+ IN SBFI-LOADED MYOCYTES

A. Iontophoretic injection of SBFI in rabbit ventricular myocytes

To our knowledge, the few studies involving fluorescent Na^+ measurements in cardiac myocytes have been carried out exclusively with the use of the membrane-permeant SBFI-AM. As discussed previously, the use of AM forms can lead to several problems, including intracellular compartmentalization and incomplete deesterification. To address these drawbacks, direct intracellular injection of the acid form of SBFI was attempted. This approach, originally used with the Ca^{2+} indicator Fura-2 (e.g. Barcenas-Ruiz and Wier, 1987; Wier et al. 1987), is generally performed through micro-injection, using a large suction pipette. Measurements of rapid Ca^{2+} transients may not be altered significantly using this dialysis technique. On the other hand, determinations of steady-state or slow changes of intracellular ion concentration are likely to be impaired due to ion diffusion into the pipette. An alternative approach was recently reported by Eley et al. (1991) who injected the dye by iontophoresis through a very small suction electrode, thereby offering the advantage of minimizing the loss of intracellular components through dialysis from the pipette. Changes of intracellular Na^+ concentration are likely to occur at

a slower rate than Ca^{2+} fluctuations and, hence, their measurement would not be possible with the dialysis technique. This makes iontophoresis the most appropriate approach for such measurements.

In this study, the development of an approach to allow the iontophoretic injection of SBFI in rabbit ventricular myocytes was attempted. As presented in Figure 3, this was found to be feasible with the injection of a relatively small negative current (-1 nA) for a short period of time (2 to 4 min). In 95% of the cells injected, the membrane potential remained the same before and after injection, thereby indicating that the current injection did not damage or alter the sarcolemmal membrane.

It is difficult to precisely determine the concentration of the injected SBFI in the myocytes cytosolic space, because the injection timeframe for each was different, depending upon how long it took to reach the desired level of fluorescence, and also since the volume and shape of individual cells is highly variable. However, it is possible to approximate the final concentration of the dye. The average injection time was about 180 sec, with a constant current of -1 nA (-1 nC/sec). Multiplying the charges per second by the average injection time, then dividing the result by Faraday's Constant, yields the total number of charges moving through the pipette tip; 1.87×10^{-12} moles. The average volume of a cardiac ventricular myocyte is in the order of 40 pl (Désilets and Baumgarten, 1986b). Approximately 35% of this volume is taken up by the mitochondria and other organelles, and is not accessible to the indicator. Dividing the previously calculated total charges, by the remaining 26 pl cytosolic volume, results in a concentration of 72 mM of

injected charge. The SBFI concentration in the pipette was 1.5 mM in an electrode solution that contained ionized salts with a total charge concentration of approximately 300 mM. Since one molecule of SBFI carries four negative charges, the dye represents about 2% of the total charges in the pipette. Even assuming that SBFI moves as freely as the other ions in the solution (e.g. K^+, Na^+ and Cl^-), the maximum concentration of the indicator in the cytosol would be 0.36 mM. Based on this conservative estimate, and the fact that each molecule of SBFI could bind to only one Na^+ ion, it can be concluded that the buffering effect of SBFI on intracellular Na^+ should be minimal.

B. Characterization and calibration of SBFI

As illustrated in Figure 2 in the Results Section, an increase of the Na^+ concentration in the bath solution resulted in a corresponding increase in the intensities of emission light for excitation wavelengths below 370 nm, and a decrease for those wavelengths above that point. This characteristic makes it possible to relate Na^+ concentrations to ratios of intensity values taken at two wavelengths on both sides of the isosbestic point, such that the calibration curve becomes independent of the concentration of the dye. Other authors used ratios of the values between 340-350 nm divided by those at 380-385 nm. After the characterization of the excitation spectrum of SBFI, it was determined that the ratio of 345 over 380 nm was optimal for the system used in this study for the following reasons. The value of 345 nm was chosen because it provided the highest light intensity at higher Na^+ concentrations. Additionally, although a wavelength higher than 380 nm could have given a better ratio, the light intensities would have been too low to be distinguishable from the background intensity levels. The ratio results varied between 2.56 in a sodium-free solution to 6.67 at 138 mM of sodium, giving an apparent dissociation constant of between 22.6 mM at the excitation wavelength of 345 nm, and 17.8 mM at 380 nm. Minta and Tsien, in their original report (1989), found an apparent dissociation constant of between 17 and 18 mM. The slight discrepancy could be accounted for by differences in experimental conditions. An important factor could be the differences in temperature, since measurements were carried out at 37°C in this study,

while those in the other were performed at 20°C. As seen in Figure 2, Panel B, equation 3 was used to fit the calibration curves.

$$[Na^+] = \frac{100C(R-R_{min})}{100(R_{100}-R)+C(R_{100}-R_{min})} \ (in\ mM) \qquad (3)$$

Applying the equation to the extracellular solution calibration yielded a value for the constant C of 28.5 mM, which is the mid-ratio concentration as defined in the Results Section. This constant represents the Na^+ concentration that will result in a ratio value corresponding to the mid-point between R_{min} and R_{max}.

Since the optical properties of aqueous solutions in the bath are different from those of cytosolic ones, intracellular calibration was required. This is the first time that the SBFI-free acid form was injected and calibrated inside cardiac myocytes. It is difficult to compare the results of the extracellular calibration found by Minta and Tsien, (1989), to the intracellular calibration findings of this study, since the apparent dissociation constant of SBFI for Na^+ in the cytosol of the cardiac myocytes was not calculated here, as excitation scans were not performed. This was due to the complexity of the scanning procedures when done on myocytes, as opposed to bath solutions, and the vulnerability of the cells to prolonged measurement protocols. These factors made it very difficult to obtain scan readings of all the required Na^+ concentrations from the same cell. Rather, equation 3 was used to fit the calibration curves, as seen in Figure 5. Two important differences between the intracellular and the extracellular calibrations were found. First, the minimum ratio measured inside the cells decreased to a value of 1.44. Secondly, the

intracellular environment appeared to cause a right shift of the calibration curve, such that the mid-ratio concentration increased to 158 mM. These effects could be partially due to differences in the viscosities of the intra- and extracellular environment (Harootunian et al., 1989), as well as to the binding of SBFI to some of the intracellular components (Donoso et al., 1992).

These differences between the intracellular and extracellular characteristics of SBFI could lead to some uncertainties with regard to the effects of changes in both intracellular Ca^{2+} concentration and pH on the properties of the indicator under different experimental conditions. However, it is unlikely that these changes would affect the intracellular calibration of the fluorescent dye during the experimental protocols designed to determine the effects of isoproterenol on $[Na^+]_i$. In the case of possible sensitivity to $[Ca^{2+}]_i$, Harootunian et al (1989) argued that this is unlikely, due to the fact that the 38 mM dissocation constant for SBFI and Ca^{2+} in saline solution is so much greater than cytosolic $[Ca^{2+}]_i$, that the affinity of SBFI to Ca^{2+} would need to change by a factor of over 10^5 to affect the fluorescent properties of the indicator in the intracellular environment. Similarly, changes in intracellular pH should have little effect on SBFI fluorescence. As described by Minta and Tsien (1989), acidification depresses the overall fluorescence of the dye, but has no effect on the ratio for pH values above five. Furthermore, the pH values in the experimental conditions of this study were unlikely to undergo important changes, considering the powerful pH regulators present in cardiac myocytes (Kaila and Vaughan-Jones, 1987; Dart and Vaughan-Jones 1992).

The apparent dissociation constant for the AM form of the indicator was calculated in cardiac myocytes by Donoso et al. (1992), who reported the value as 29.3±2.7 mM. Though they did not perform intracellular excitation scans, Donoso et al. were still able to do this because their 340 nm light intensity was constant throughout the calibration, which was not the case with the intensity at 345 nm used in this research project. The ratio changes in their study were due to shifts in the light intensities at an excitation wavelength of 380 nm. The fact that the light intensity changes at 380 nm were not accompanied by simultaneous but opposite changes at 340 nm, indicates that an intracellular scan would not have produced an isosbestic point, i.e. there would be no intersection of the lines on the graph representing the excitation scans at different Na^+ concentrations. This could have been due to incomplete deesterification of the SBFI-AM within the cell since, in the same study, extracellular excitation scans, using the acid form of the indicator, did yield a crossover of the scan tracings at different Na^+ concentrations around the isosbestic point. In contrast to Donoso et al. (1992), and referring to Figure 4, Upper Panel of the current study, where the acid form of the indicator was injected into the cell, it is evident that varying the Na^+ concentration produced simultaneous and opposing changes in light intensities at both excitation wavelengths, 345 and 380 nm. This suggests that the intracellular optical properties of the AM and the acid forms are different, which indicates that the deesterification of the AM form was incomplete, since full deesterification would leave only the acid form of the indicator within the cytosol of the myocyte. In fact, Borzak et al. (1992) did an intracellular scan using the AM form,

which not only failed to show a crossover at varying Na^+ concentrations, but also exhibited an inverse excitation spectra compared to those of the acid form in the extracellular solutions. As noted in the Introduction, the incomplete deesterification of fluorescent indictors is a major limitation to their use in the study of changes in the concentration of intracellular ions. The use of iontophoresis to inject the acid form of SBFI into the cytosolic space of myocytes directly, may provide the means to overcome this drawback.

In this study, the membrane potential ranged from –4.6 to -7.8 mV in solutions containing 0 mM Na^+ and 100 mM Na^+, respectively, during the intracellular calibration of the fluorescent dye (see results). This could be an indication that the $[Na^+]_i$ was not equal to the $[Na^+]_o$ during the calibration. Since the membrane potential was not equal to 0 mV, it follows that a negative potential would create an electrical gradient favouring the movement of sodium toward the interior of the cell, which could lead to an underestimation of the $[Na^+]_i$. According to Nernst's equation, around the previously mentioned -4.6 to -7.8 mV membrane potential range, the $[Na^+]_i$ could be greater than the $[Na^+]_o$ by a maximum factor of to 20% to 33% respectively. Chloride was the major anion used in the calibration solution. As this anion can move relatively freely compared to the intracellular negatively charged proteins, it creates a Donnan effect, which could account for the negative potential. To avoid such an effect, further calibrations should be performed with an impermeable negative organic anion, instead of Cl^-. Another possible explanation for the unequal diffusion of ions, reflected in the observed negative membrane

potential, could be the presence of some uninhibited ion active transport mechanisms. If replacing Cl^- with an impermeable negative organic anion proves to be unsuccessful in nullifying the observed negative membrane potential, an ATP blocker could be used to inhibit any remaining cellular active transporters. Despite this possible calibration deficiency, the relative changes in $[Na^+]_i$ could still be monitored with the method described in the current research project.

In this study, during the intracellular calibration of SBFI, two types of ionophores, gramicidin and amphotericin B, were used to produce transmembrane pores in order to achieve equal distributions of intracellular and extracellular ions (see Introduction for the rationale for using these ionophores). It also might have been advisable to use the ionophore monensin, with its much higher conductivity to Na^+ (Pressman, 1976), in addition to gramicidin and amphotericin B, in order to further decrease the time required to reach a steady-state of $[Na^+]_i$. This was confirmed by Lee and Levi (1991), who calibrated SBFI-AM in isolated single rabbit cardiac myocytes. They reported an improved equilibration of $[Na^+]_i$ and $[Na^+]_o$, and an accelerated rate of ratio response to changes in extracellular concentration, by using monensin in addition to gramicidin. They concluded that the addition of monensin is likely to give a more correct estimate of the $[Na^+]_i$. There is a potential problem involved with the use of monensin, however, as it does not simply increase Na^+ conductivity by transporting Na^+ ions across the cell membrane, but by exchanging them for protons (Pressman, 1976). This could change the intracellular hydrogen ion concentration, which could, among other things, create a pH gradient,

thereby preventing the passive distribution of Na^+ across the cell membrane, through the activity of the Na-H exchanger and the Na-HCO_3 symport. This problem might be resolved through the simultaneous use of another ionophore, nigericin, which exchanges protons for K^+ (Pressman, 1976; Harootunian et al., 1989), thereby helping to negate any pH gradient which might have been created, while simultaneously enhancing the K^+ conductance. In fact, the combination of monensin and nigericin has been used for the calibration of SBFI in other tissue preparations (Harootunian et al., 1989; Lee and Levi, 1991).

Once these suggested improvements are implemented, the calibration method described in this study, and the equation derived from it (equation 3), will be useful in performing fast intracellular calibrations for SBFI. It is impossible to reach the saturation point of the dye without changing the osmotic properties of the solutions. This equation provides a means to calibrate for the fluorescent indicator without the need to know the value of the ratio at the saturation point of the dye, i.e. R_{max}.

C. Advantages of combining two techniques: Measuring fluorescent changes under voltage-clamp conditions

As noted in the Introduction, studying the pump current without monitoring the changes in $[Na^+]_i$ leads to controversies in interpreting the results of such measurements since they were performed under unphysiological conditions i.e. they required extensive use of pharmacological blockers. Conversely, measuring $[Na^+]_i$ without controlling the membrane potential could lead to difficulties in interpretation of the results because of the possible effects of the changes of membrane potential on the movement of Na^+. The technique of using an ion-selective electrode to monitor $[Na^+]_i$, combined with another electrode to monitor current, is difficult and almost impossible on single cells. The approach used in this study, combining fluorescent measurement of the $[Na^+]_i$ with voltage-clamping, provides an easier alternative. There were some technical difficulties obtaining stable simultaneous measurements from both the fluorescent and electrical recordings. This problem, which led to discrepancies in terms of the numbers of reported cells being measured, could be overcome through improvements in equipment, skill levels and overall experimental design. For example, the use of solenoid magnetic valves, instead of mechanical valves, for switching between superfusion solutions markedly reduced cell injury, and resulted in a decreased discrepancy in the number of reported cells. This is demonstrated in Figure 17, which shows more discrepancy in the Ca^{2+} and STR groups, where mechanical switching was employed, than in the EGTA and BAPTA groups, where

the solenoid valves were used. In any case, and as discussed in the next section (Effect of Isoproterenol on the Na-K Pump), there was indeed a good correlation between the changes of $[Na^+]_i$ and shifts of transmembrane ion currents.

Monitoring the $[Na^+]_i$ under voltage-clamp conditions should also allow the quantification of the active Na^+ efflux, through relating the changes in $[Na^+]_i$ to the transmembrane current, i.e. the amount of charge crossing the cell membrane, over the time period of the $[Na^+]_i$ change. Although this quantification was not performed in this study, the potential of the technique to achieve such quantification is addressed further on in the Discussion Section.

Nevertheless, using the combined approach, the average steady-state $[Na^+]_i$ was found to be 6.4±1.8 and 13.0±1.4 mM in 2 and 0.8 mM Ca^{2+}, respectively. An increase of $[Na^+]_i$ in low extracellular Ca^{2+} concentrations is a well known phenomenon (Chapman et al. 1986), and is related to an increased Na^+ leak in solutions containing low Ca^{2+} concentrations. In any case, these values, even after correcting for possible underestimation due to the Donnan effect, would be lower than those measured with ion-selective microelectrodes in rabbit preparations maintained under comparable conditions. Thus, Shattock and Bers (1989) obtained a value of 9.5 mM in quiescent ventricular muscles maintained in 2 mM Ca^{2+}, while Désilets and Baumgarten (1986a,b) obtained a value of 12 mM in isolated ventricular cells bathed in 2.5 mM Ca^{2+}. The differences in these values could be due to the problem of an incomplete membrane seal around the ion-selective microelectrodes, which could create a Na^+ leak. On the other hand, Lee and Levi

(1991) measured a $[Na^+]_i$ value of 3.8±0.2 mM in rabbit isolated myocytes loaded with SBFI-AM and superfused with solutions containing 2 mM Ca^{2+}. This value is remarkably low considering the fact that the cells were studied at 30°C which should, if anything, cause an increase of intracellular Na^+ levels. Two factors could have contributed to this low value. First, as noted by Lee and Levi, it was difficult to ascertain that the Na^+ was equally distributed during the calibration procedures. The second and more important factor is the possibility that a large portion of SBFI-AM was distributed in intracellular compartments, thereby giving erroneous values of the cytosolic free-Na^+ concentration. In fact, it has been reported that as much as 50% of SBFI-AM in rat ventricular myocytes was located in the mitochondria (Donoso et al., 1992; Borzak et al., 1992), and that the mitochondrial Na^+ concentration is lower than that of the cytosol (Donoso et al., 1992).

EFFECT OF ISOPROTERENOL ON Na-K PUMP

A. Effect on steady-state $[Na^+]_i$

Under steady-state conditions, and in the presence of 2 mM Ca^{2+}, there was no significant effect on the $[Na^+]_i$ or the transmembrane current after exposure to 0.5 μM of isoproterenol (Figure 6). This observation disagrees with several studies that showed a decrease of intracellular Na^+ levels in Purkinje fibres (Lee and Vassalle, 1983; Pecker et al., 1986), ventricular trabeculae (Wasserstrom et al., 1982) and isolated ventricular myocytes (Désilets and Baumgarten, 1986b). The reason for this discrepancy is not clear, but may be related to the level of steady-state Na^+ present in the preparations. Thus, in the study of rabbit ventricular myocytes (Désilets and Baumgarten, 1986b), steady-state $[Na^+]_i$ was of the order of 12 mM, a value similar to that reported for canine Purkinje fibres exposed to catecholamines (Wasserstrom et al. 1982; Lee and Vassalle, 1983; Pecker et al., 1986). In this study, however, the effects of isoproterenol were tested in cardiac myocytes with intracellular Na^+ concentration levels of about 6 mM, which may account for the lack of response.

The discrepancy in results with those of the other studies led to the hypothesis that the effect of isoproterenol may be dependent on the intracellular concentration of Na^+ ions. An attempt was made to test this hypothesis by studying the effects of isoproterenol in cardiac myocytes with augmented intracellular Na^+ concentration.

B. Effect of gramicidin on intracellular Na^+

In order to increase the Na^+ permeability and, hence, intracellular Na^+ levels, the monovalent cation-selective ionophore gramicidin was used (Finkelstein and Andersen, 1981; Harootunian et al., 1989). Initially, the objective was to use the ionophore to augment the $[Na^+]_i$ to new steady-state levels. This proved to be more difficult than anticipated for several reasons.

At higher concentrations, i.e. greater then 30 nM, gramicidin tends to elevate the $[Na^+]_i$ to a level incompatible with cell survival. A possible explanation may be related to the mechanism by which gramicidin interacts with the cell membrane, one which is different than that of most other ionophores. Gramicidin molecules enhance monovalent cationic conductivity by inserting themselves across the full thickness of the lipid bilayer of the cell membrane, and subsequently becoming stationary ion-conducting channels. This causes the Na^+ movement across the membrane to take place at a far greater rate than would be the case with most other ionophores (Pressman, 1976). However, at lower concentrations, i.e. less than 30 nM, two difficulties were encountered. First, the onset and the rate of Na^+ increase varied extensively between cells. Secondly, and more importantly, obtaining higher levels of steady-state Na^+ was not possible because the $[Na^+]_i$ values kept increasing, independent of the concentration of gramicidin. This could be related to the observation that gramicidin might require a great deal of time to reach equilibrium between its two concentrations; that of the extracellular solution, and that within the

cardiac myocyte membrane (see Results Section). This could be demonstrated by the gradual and continuous inward shift of the transmembrane current during exposure of the cells to the ionophore.

Also, the Na^+ leak created by gramicidin appeared to be highly sensitive to the presence of extracellular Ca^{2+}. Thus, as demonstrated in Figures 9 and 11 in the Results Section, the removal of extracellular Ca^{2+} caused a decrease of the gramicidin-induced Na^+ influx by a factor of 2.65, despite the fact that the gramicidin concentration was three times higher in the absence of extracellular Ca^{2+} than in its presence. This directly indicates that the incorporation of gramicidin into the cardiac myocyte membrane is greatly impaired by the removal of Ca^{2+} ions, which makes it even more difficult to reach a new higher level of $[Na^+]_i$.

In any case, despite the fact that it was not possible to use the ionophore gramicidin to augment the $[Na^+]_i$ to new steady-state levels, the presence of a continuous gramicidin-induced increase in the intracellular Na^+ concentration was useful in studying the qualitative effects of isoproterenol.

Finally, it should be mentioned that the increase in $[Na^+]_i$ induced by gramicidin rapidly lead to cell instability and spontaneous contractions, which became severe with isoproterenol. This is presumably due to an increase in intracellular free-Ca^{2+} concentration following inhibition of Ca^{2+} efflux through the Na-Ca exchange (see Wier, 1991). These oscillations could be attenuated, however, by the use of ryanodine during the exposure to gramicidin (see Introduction for more information about the properties of ryanodine).

C. Effect of isoproterenol on gramicidin-induced Na^+ influx

As shown in Figures 8 and 9, in the Results Section, isoproterenol caused a reversal of the gramicidin-induced $[Na^+]_i$ increase, together with a clear outward shift of the transmembrane current. These observations are compatible with the concept of stimulation of the Na-K pump by the β-agonist, as opposed to a decrease of Na^+ leak. Unless it is assumed that the gramicidin-induced Na^+ influx is somehow inhibited by isoproterenol, the observation that this influx was actually reversed in the presence of the β-agonist suggests that active Na^+ efflux was stimulated, as isoproterenol has been found to increase the Na^+ leak in myocardial tissue (Kassebaum and Van Dyke, 1966; Brown and DiFrancesco, 1980; Hougen et al., 1981; Désilets and Baumgarten, 1986b). Furthermore, the relative effects of isoproterenol on $[Na^+]_i$ rate of rise and the transmembrane current were not altered by the removal of external Ca^{2+} (Figure 17). This observation rules out the possibility that these effects are due to isoproterenol-induced active extrusion of Na^+ ions in exchange for extracellular Ca^{2+} through the Na-Ca exchanger. As such, the stimulation of the Na-K pump appears to be the major mechanism accounting for the observed changes. This conclusion was confirmed by the use of strophanthidin, which completely blocked both the isoproterenol-induced decrease of $[Na^+]_i$ and the outward shift of transmembrane current (Figures 12-14). Altogether, these results strongly support the conclusion that the Na-K pump is stimulated by isoproterenol.

It is interesting to note that the effect of isoproterenol on both Na^+ influx and transmembrane current appears to be closely related to the level of intracellular Na^+ concentration, as presented in Figure 18. This explains why isoproterenol failed to induce detectable changes under steady-state conditions in the absence of gramicidin, and indicates that the stimulation of the pump occurs through an increase of the maximum turnover rate (V_{max}), as opposed to an increase of the pump affinity for intracellular Na^+. In the latter case, one would have expected a decrease of isoproterenol effect with high $[Na^+]_i$, where the pump would have been close to maximal activation by intracellular Na^+. The possibility that the stimulation of the pump activity occurs through an increased affinity for extracellular K^+ ions, as suggested by experiments on bullfrog atrial bundles (Akasu et al., 1978) and on canine purkinje fibres (Gadsby, 1983), is also questionable. The experiments in the current study were performed at 5 mM of extracellular K^+, a concentration that is probably close to the pump's saturation level, considering that the K^+ concentration required for half maximal activation of the pump is about 1 mM (Gadsby, 1980; Eisner, 1981b; Glitsch et al., 1982; Falk and Cohen, 1984). Also, Akasu et al., (1978) and Gadsby, (1983), performed their experiments in multi-cellular preparations, as opposed to the single-cell preparations which were used herein. Since the effects of increased K^+ conductance by isoproterenol, and the subsequent increase of extracellular K^+, would probably be far less significant in single cell preparations, the suggestion that the effects of the β-agonist on the Na-K pump which have been observed in this study are due to an augmentation of extracellular K^+, appears to be unlikely. In fact, Désilets and

Baumgarten (1986b) demonstrated that the stimulatory effect of isoproterenol on the Na-K pump in isolated rabbit single cardiac myocytes was not affected by increasing the extracellular K^+ concentration to 15 mM. However, their experiments were not performed under voltage-clamp conditions, which made it difficult to distinguish between the effects of voltage fluctuation, and the mechanisms involved in isoproterenol activation of the pump. It is possible to verify the question of K^+ involvement in the stimulation of the Na-K pump by the β-agonist by measuring the isoproterenol-induced changes in intracellular Na^+ under voltage clamp conditions in high extracellular K^+ concentrations, to ensure that the pump is completely saturated with K^+. Further stimulation of the Na-K pump by the β-agonist under these conditions, would preclude the possibility that the pump is stimulated through an isoproterenol increased conductance of K^+. The conclusion that β-agonists increase the maximal turnover rate of the Na-K pump is in agreement with the results of Gao et al. (1992), who demonstrated that the relative change of pump current induced by isoproterenol appeared to be independent of both extracellular K^+ concentration and the Na^+ concentration present in their dialysing pipette.

Although the creation of a steady, progressive increase in $[Na^+]_i$ by gramicidin was useful in determining the qualitative effects of isoproterenol on the Na-K pump, this procedure did not provide the means to obtain an accurate quantitative evaluation of these effects. A more accurate quantitative analysis of the relative isoproterenol-induced change in the Na-K pump activity could have been achieved if the $[Na^+]_i$ was elevated to a higher steady-state level. As noted previously, and as shown in Figure 18, the magnitude of the

effects of isoproterenol on the gramicidin-induced Na^+ influx was dependent upon the level of intracellular Na^+ concentration. This continuous and progressive Na^+ leak made it difficult to compare the magnitude of the decrease in $[Na^+]_i$ due to isoproterenol, with a reference value of $[Na^+]_i$ in the same cell, as there was no steady-state Na^+ value to compare with. Exposure of the cell to gramicidin also caused a continuous inward shift of the transmembrane current, as noted in the previous section (Section B). This also made it difficult to use the measurement of the changes in transmembrane current, to quantify the relative isoproterenol-induced change in the Na-K pump activity. In the absence of such an inward shift, it would have been possible to quantify the value of the isoproterenol-induced outward shift against the basal current value of the Na-K pump, induced by a pulse of strophanthidin, within the same cell.

Nevertheless, it is possible to estimate the relative change in the Na-K pump activity by comparing the average $[Na^+]_i$ changes observed in the presence of isoproterenol and strophanthidin, to those observed in the presence of isoproterenol alone. For instance, the average rate of rise in $[Na^+]_i$ measured in the presence of strophanthidin and gramicidin was 6.5 mM/min (Figure 14), while that measured in gramicidin alone was 1.1 mM/min, giving a net active Na^+ efflux of 5.4 mM/min. Under the same conditions without strophanthidin, isoproterenol caused a decrease in the average rate of rise in $[Na^+]_i$ of 8.0 mM/min (Figure 17), thereby representing a 148% increase in the active Na^+ efflux rate. One could also estimate the effect of isoproterenol on the Na-K pump activity by calculating the changes of transmembrane current, as indicated in Figures 12-14. Thus, the

average current shift induced by strophanthidin was -104 pA, while that induced by isoproterenol was 58 pA, thereby representing a 56% increase in pump current. A comparison of the results of the two approaches, i.e. the changes in the $[Na^+]_i$ versus those of the transmembrane current, yields a relatively large difference in values for this estimated isoproterenol stimulation of the Na-K pump. There are several factors that might explain this discrepancy. First, the estimates were derived from average values measured from different groups of cells. Second, the 56% stimulation derived from the current traces is likely to be underestimated, as discussed previously, due to the continuous decline of the gramicidin-induced transmembrane current, which would partially mask the outward shift induced by isoproterenol, and cause an overestimation of the strophanthidin-induced inward shift. Interestingly, from measurements performed using an ion-selective microelectrode, Désilets and Baumgarten (1986b) reported an increase of 90% in the active Na^+ efflux rate in isolated rabbit cardiac myocytes upon exposure to the β-agonist, a value between the two percentages for the isoproterenol-induced stimulation of the Na-K pump estimated in this study.

Nevertheless, the magnitude of the isoproterenol stimulation of the Na-K pump in the presence of a gramicidin-induced Na^+ leak was remarkable, considering the apparent absence of any effects under steady-state conditions. Even the likely underestimated value of 56% stimulation of the Na-K pump activity is much more important than the maximum value of 25% recently reported from measurements of pump current in guinea-pig isolated myocytes (Gao et al., 1992).

Accurate quantification of the relative isoproterenol-induced change in the Na-K pump activity could be achieved by finding an alternative experimental approach to increase the intracellular Na^+ concentration to a new steady-state level, rather than augmenting it by the continually rising, gramicidin-induced Na^+ leak, which was the procedure used in this study. There are a number of possible alternatives by which this could be accomplished.

One potential alternative methodology would involve the use of site 2 Na channel toxins, such as batrachotoxin (BTX), veratridine or aconitine. These lipid-soluble toxins would help to elevate the $[Na^+]_i$ by favouring the open state of the activation (m) gate of the Na channel, while simultaneously depressing the closure of the inactivation (h) gate (Catterall, 1991; Fozzard and Hanck, 1991). The advantage of using these compounds is that, as it should be possible to study their dose response patterns, the most appropriate concentration of toxin to use in the extracellular superfusion solution can be determined, thereby allowing for optimal control of the level of $[Na^+]_i$. The effects of these toxins are voltage dependent, and although more effective under depolarized membrane potentials (Fozzard and Hanck, 1991), they could also be used within a wide range of voltage-clamp conditions. An additional advantage could be gained through the removal of the divalent cations from the superfusion solution, which would allow the Na^+ to pass through the Ca^{2+} channels (Chapman et al., 1986), and contribute to the elevation of $[Na^+]_i$ provided by the site 2 Na channel toxins. Since the effects of Ca^{2+} on the Na-K pump, and the role of Ca^{2+} in the isoproterenol activation of the pump, are not well defined as yet, extra care should

be taken when interpreting the results after the removal of the divalent cations from the extracellular solution.

Another possible alternative which could provide a quantitative analysis of the effects of isoproterenol on the Na-K pump, would be to augment the $[Na^+]_i$ by inhibiting the pump, through the removal of extracellular K^+, and subsequently reactivating the pump through the reintroduction of K^+ both in the presence, and absence, of isoproterenol. As noted previously, this technique would allow for quantification of the active Na^+ efflux by relating the decrease in the $[Na^+]_i$ upon reactivation of the pump, to the magnitude of the accompanying outward shift in the transmembrane current. It would also allow the measurement of the isoproterenol-induced change in the Na-K pump activity, through the evaluation of the effect of the β-agonist on the time course of the intracellular Na^+ concentration level recovery, and the related changes in transmembrane current, after the reintroduction of extracellular K^+. In fact, Désilets and Baumgarten (1986b) employed a similar technique to assess the effects of isoproterenol on the Na-K pump in isolated cardiac myocytes using an ion-selective electrode, although it should be noted that their study was not performed under voltage-clamp conditions.

Overall, the results discussed in this section demonstrate that ß-agonists can have direct and profound stimulatory effects in rabbit ventricular myocytes. The physiological significance of this phenomenon may be related to the consequent enhancement of the Ca^{2+} and H^+ extrusion, through activation of the Na-Ca and Na-H exchangers, and the Na-HCO_3 cotransporter. Under adrenergic drive, intracellular Ca^{2+} and H^+ are expected to

increase following enhanced contractile and metabolic activity. A simultaneous decrease of $[Na^+]_i$ through stimulation of the Na-K pump would activate the unloading of these potentially harmful intracellular ion species. In that regard, the fact that ß-agonist stimulation of the pump increases as the $[Na^+]_i$ rises, further points to the protective feature of this stimulation.

D. Effect of Ca^{2+} on isoproterenol-induced changes of Na-K pump activity

As mentioned above, the removal of extracellular Ca^{2+} significantly affected the capability of gramicidin to cause an increased Na^+ leak. However, this manoeuvre did not appear to alter the stimulation of the Na-K pump activity by isoproterenol. As described in Figure 17, the changes of Na^+ influx, and the outward shift of the transmembrane current, were comparable in both the presence and absence of extracellular Ca^{2+}. In addition, the amplitude of these isoproterenol-induced changes correlated to the $[Na^+]_i$ in the same manner, whether or not Ca^{2+} was present in the bathing solution (Figure 18).

In these experiments, extracellular Ca^{2+} was removed just prior to SBFI injection, such that the level of intracellular free-Ca^{2+} was likely to remain relatively high, as demonstrated by the tendency of a substantial portion (40%) of the myocytes to spontaneously contract in the presence of gramicidin and isoproterenol. In order to study the hypothetical involvement of intracellular Ca^{2+}, cells were further preincubated for at least two hours in Ca^{2+}-free solutions containing 0.5 mM EGTA and 25 μM of the Ca^{2+} chelator BAPTA-AM (Tsien, 1980). Unfortunately, as noted in the Results Section, three of the nineteen BAPTA-AM preincubated cells showed contractile activity, an indication that the intracellular Ca^{2+} was not completely chelated. Although this is a smaller proportion than the previously mentioned 40%, where the extracellular Ca^{2+} was removed, and despite the fact that these three beating cells were excluded from the analysis, their activity calls into question the claim that intracellular Ca^{2+} was completely chelated in the

other 16 cells. It also makes it uncertain if the preincubated cells contained any BAPTA at all at the time of their exposure to isoproterenol. Incomplete deesterification of the BAPTA-AM could account for a substantial loss of the chelator to the extracellular superfusate, once the cells have been removed from the preincubation solution. Nevertheless, as will be further discussed, the fact that the cells which were incubated in both EGTA and BAPTA-AM for a prolonged period of time responded to isoproterenol differently than those which were incubated in EGTA shortly before their injection with SBFI, strongly indicates that the intracellular Ca^{2+} concentration was substantially reduced. This difference in response could be due to the prolonged incubation in a Ca^{2+} free solution, rather than the effect of BAPTA-AM.

Preincubation of the cells in BAPTA-AM resulted in a strong attenuation in the effects of isoproterenol on both the Na^{+} influx and the transmembrane current (Figures 15-17). This inhibition, which did not appear to be affected by membrane voltage (Figure 16), also occurred independent of the intracellular Na^{+} level present at the time of the introduction of isoproterenol (Figure 18). It can therefore be concluded that the complete or, at least, partial removal of Ca^{2+} from the intracellular milieu, unlike its removal from the extracellular solutions, caused an inhibition of the stimulatory effect of isoproterenol on the Na-K pump. This conclusion supports the results reported by Gao et al. (1992) from a study done on guinea-pig ventricular myocytes, who found an isoproterenol-induced increase of the strophanthidin-sensitive current only in cells having free-Ca^{2+} concentrations above 150 nM. As mentioned earlier, the major difference between the

present study and that of Gao et al. (1992) is the magnitude of the effect of isoproterenol which, in their case, stimulated the pump by not more than 25%, and occurred only at very high levels of intracellular Ca^{2+} (>1 μM). Although the difference in species could be a factor, this relatively small effect could also be explained by the fact that Gao et al. studied the pump activity by examining the transmembrane current alone, such that the effect of isoproterenol could have been partially masked by a parallel effect of the β-agonist on other ion conductances than that of the Na-K pump. It is interesting to note that the isoproterenol in their study caused an inward current shift in guinea-pig ventricular myocytes voltage-clamped at -60 mV, while an outward current shift was observed in the present research project conducted on rabbit myocytes voltage clamped at -70 mV.

Although the present study demonstrated the important involvement of intracellular Ca^{2+} in the isoproterenol-induced stimulation of the Na-K pump, the experimental design could not provide a detailed understanding of the means by which Ca^{2+} affects this stimulation. As such, the mechanism underlying the stimulatory effect of isoproterenol and its dependency upon intracellular Ca^{2+} can only be of a speculative nature. In a manner similar to that proposed by Berthon et al. (1985) to explain the effect of α-agonists in hepatocytes, isoproterenol, through ß-activation, could act by removing the inhibition on the Na-K pump that is caused by intracellular Ca^{2+} ions. Conversely, Gao et al. (1992) suggested an inhibitory effect of isoproterenol on the Na-K pump, that is removed by Ca^{2+}. Gao et al. also proposed a direct phosphorylation of the Na-K ATPase, and a consequent interaction with intracellular Ca^{2+}. It should be noted that, although the

removal of intracellular Ca^{2+} by BAPTA-AM seemed to result in higher values of $[Na^+]_i$, this is unlikely to be due to direct inhibition of the Na-K pump by low $[Ca^{2+}]_i$. It is more probably a result of the increased Na^+ leak through the Ca^{2+} channels in the absence of the divalent cation (Chapman et al., 1986), as previously mentioned.

To verify which of the proposed theories best explains the mechanism by which Ca^{2+} is involved in the stimulation of the Na-K pump by isoproterenol, the direct effects of intracellular Ca^{2+} on the pump activity should be studied. In the former case, the removal of Ca^{2+} should cause a stimulation of the Na-K pump in the absence of ß-agonists, but not in their presence, while, in the second hypothesis, the removal of Ca^{2+} should result in an inhibition of the Na-K in the presence of ß-agonists, but not in their absence. As the removal of intracellular Ca^{2+} proved to be more difficult than anticipated, it is important to perform the proposed studies in a manner which will ensure the absence of this ion species in the cytosol of the cell. This could be accomplished through the use of either of the following procedures.

One proposed methodology would be to attempt to inject the BAPTA directly into the cytosol of the cardiac myocyte from the pipette along with, and at the same time as, the SBFI is introduced through iontophoresis. The advantage of this method is that, while most Ca^{2+} chelators also affect other divalent cations, BAPTA has a high affinity for Ca^{2+}, a high selectivity over Mg^{2+} and is not affected by intracellular H^+ concentration (Tsien, 1980; Blinks, 1991).

Another approach would be to first test the effects of isoproterenol in the presence of Ca^{2+}, remove the extracellular Ca^{2+} by superfusing the cell with a solution containing EGTA, and then attempt to deplete the intracellular Ca^{2+} store by exposing the cell to a pulse of caffeine, which causes the release of Ca^{2+} from the sarcoplasmic reticulum by opening its Ca^{2+} channels (Rousseau et al., 1986). The effect of isoproterenol is then tested again with the cell under zero Ca^{2+} conditions. The advantage of this method is that it will enable the comparison between the effects of isoproterenol in the presence and absence of Ca^{2+} in the same cell. The disadvantage of this approach is that it might also involve the chelation of Mg^{2+} by EGTA, in addition to Ca^{2+}.

To determine which of the suggested approaches would be the most effective method for the removal of Ca^{2+}, the intracellular concentration of the divalent cation should be measured in cells treated in a similar manner as that of the proposed experimental methodologies. This could be accomplished through the use of a Ca^{2+} fluorescent indicator (e.g. FURA-2), which could be injected iontophoretically from the micro-electrode into the cardiac myocyte, as described by Eley et al. (1991).

CONCLUSION

In conclusion, this study demonstrates that the Na^+-sensitive fluorescent indicator SBFI can be successfully and reliably injected into ventricular myocytes by iontophoresis. The results confirm that the properties of the dye change substantially in the intracellular environment, thereby necessitating careful calibration of the indicator in the myocytes. In that regard, the calibration technique could be improved to ensure that Na^+ is equally distributed across the cell membrane. To accomplish this, one approach would consist of replacing Cl^- with impermeant anions in the bathing solution, in order to abolish the observed Donnan potential.

SBFI was used as a tool to measure intracellular Na^+ in order to study the effects of isoproterenol on the Na-K pump activity in isolated single rabbit cardiac myocytes under voltage-clamp conditions.The results herein have demonstrated that the ß-adrenergic agonist can act as a powerful stimulant of the Na-K pump. The resulting stimulation, which appears to occur directly through an increase of the maximal turnover rate of the pump, increases with the level of intracellular Na^+, and is strongly attenuated by the removal of Ca^{2+} from the intracellular milieu.

Future experiments could take several directions. The effect of intracellular Ca^{2+} on the Na-K pump activity should be determined directly. The mechanism of the isoproterenol-induced stimulation of the pump, and its dependency on intracellular Ca^{2+}, should be further investigated by examining the cAMP pathway. The effect of direct

activators of adenylate cyclase (forskolin) or permeant cAMP analogs could therefore be studied. Finally, the technique developed in this study to measure intracellular Na^+ in voltage-clamped myocytes represents a powerful tool for the direct study of Na^+ influx mechanisms and their possible hormonal regulation.

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APPENDICES

APPENDIX 1. CHEMICALS

Abbreviation	Chemical name	Source
	l-aspartic acid	BDH
$CaCl_2$	calcium chloride	BDH
	collagenase CLS II	Worthington
	creatine	Sigma
K_2-Creat.	creatine phosphate dipotassium salt	Calbiochem
	D-glucose	BDH
DMSO	dimethylsulfoxide	Sigma
K_2-ATP	dipotassium adenosine 5' triphosphate	Sigma
Na_2HPO_4	di-sodium hydrogen orthophosphate	BDH
EDTA	ethylenediaminetetraacetic acid	BDH
EGTA	ethylene glycol-bis(β-aminoethyl ehter) N,N,N',N'-tetraacetic acid	Sigma
GSH	glutathione	Sigma
Hepes	(N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfo- nic acid])	Sigma
HCl	hydrochloric acid	BDH
Mg-ATP	magnesium adenosine 5' triphosphate	Sigma
$MgCl_2$	magnesium chloride	BDH
$MgSO_4$	magnesium sulfate	BDH
KCl	potassium chloride	BDH

Chemicals (continued)

Abbreviation	Chemical name	Source
KOH	potassium hydroxide	BDH
KH_2PO_4	potassium phosphate	J.T. Baker
$NaHCO_3$	sodium bicarbonate	Fisher
NaCl	sodium chloride	BDH
NaH_2PO_4	sodium dihydrogen orthophosphate	BDH
NaOH	sodium hydroxide	BDH

APPENDIX 2. PHARMACOLOGICAL AGENTS

Name	Source
amphothericin B	Sigma
gramacidin	Sigma
(-)isoproterenol	Sigma
ouabain	Sigma
ryanodine	Calbiochem
strophanthidin	Sigma
taurine	Sigma
BAPTA/AM	Calbiochem
SBFI, free acid	Molecular Probes

APPENDIX 3. EQUIPMENT

Type	Manufacturer
Epifluorescent inverted microscope	Nikon Diaphot-DM
Solenoid electrical valve	General Valves
Voltage - clamp amplifier	Axon Instruments, Axoclamp-2A
Timer/stimulator	Winston Electronics, A-65
Video-cassette recorder	Vetter 420-B
Spectrofluorometer	Spex Industries, Fluorolog CM system
Oscilloscope	Gould Inc.

APPENDIX 4. SOFTWARE

Name	Producer
ASYST Software	Keithly Instruments
CoStat, CoDraw, CoPlot	CoHort Software
DM3000 software	Spex Industries
Systat version 5.03	Systat Inc.