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Effects of ATP on specific [^{3}H](+)-isradipine binding in rat ventricular cardiomyocytes

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Abstract

In heart membranes, specific [^{3}H](+)-isradipine binding is reduced in membranes from ischemic hearts and by adding 1 mM ATP at low Ca^{2+} concentrations (1 μM). We investigated if ATP affected specific [^{3}H](+)-isradipine binding in intact rat ventricular cardiomyocytes. Reducing intracellular ATP by 2 h hypoxia (N_2 gas) and glucose-free buffer with 1 mM CN^-, did not affect density or dissociation constant of [^{3}H](+)-isradipine binding in cardiomyocytes at extracellular 30 mM K^+. Extracellular 10 mM ATP inhibited binding in cardiomyocytes by 90% and 50%, respectively, in 30 mM and 120 mM K^+ buffer with Ca^{2+} and Mg^{2+}. Omitting Ca^{2+} and Mg^{2+} from the buffer had no effect on the binding inhibition of ATP. Hence, in cardiomyocytes, reducing intracellular ATP has no effect on specific [^{3}H](+)-isradipine binding, whereas high extracellular ATP in the presence of Ca^{2+} and Mg^{2+} inhibits binding. Apparently, ATP effects on binding differ in cardiomyocytes and membranes.

Keywords: ATP; Ca^{2+} channel antagonist; Cardiomyocyte; CN^-; Hypoxia; Ca^{2+} channel, L-type

1. Introduction

Sarcolemmal voltage-operated L-type Ca^{2+} channels mediate Ca^{2+} influx needed for excitation and contraction of cardiomyocytes. Several observations indicate that ATP influences cardiac L-type Ca^{2+} channels. Physiological levels of intracellular ATP (1 mM) are necessary to prevent run-down of L-type Ca^{2+} channels current in isolated cardiomyocytes (Belles et al., 1988). Ischemia, a process reducing intracellular ATP, decreases the binding density of Ca^{2+} antagonists to L-type Ca^{2+} channels in heart membranes (Gu et al., 1988) maybe reducing Ca^{2+} influx as suggested by Sperelakis and Schneider (1976). Sudden rises in intracellular ATP increase L-type Ca^{2+} channel current (Taniguchi et al., 1983; O'Rourke et al., 1992). Physiological levels of extracellular ATP (1 μM) increase L-type Ca^{2+} channel current (Vassort et al., 1992), whereas much higher concentrations (1 mM) decrease L-type Ca^{2+} channel current (Goto et al., 1976).

Previously, it has been shown that high concentrations of ATP, GTP and stable GTP analogs inhibited specific binding of the Ca^{2+} channel antagonist, [^{3}H](+)-isradipine, to L-type Ca^{2+} channels in membranes and cell homogenates from rat hearts at low ($\sim$ 1 μM) concentrations of Ca^{2+} and Mg^{2+} (Glossmann et al., 1982; Stokke et al., 1996). ATP inhibited specific 0.12 nM [^{3}H](+)-isradipine binding *in membranes* completely with a slope factor of one and with 50% inhibition at 0.4 mM ATP. The inhibition of binding by stable GTP analogs was present at low (1 μM), but not at high (1 mM) Ca^{2+} and Mg^{2+} concentrations.

In order to clarify effects of ATP on cardiac L-type Ca^{2+} channels, the aim of this study was to investigate if depletion of intracellular ATP by hypoxia and energy/substrate deprivation affects specific [^{3}H](+)-isradipine binding in intact isolated cardiomyocytes, and if extracellular ATP mimics effects of ATP on isolated membranes. We used the same protocols of hypoxic incubation (2 h incubation in N_2 gas *and* glucose-free buffer with or without 1 mM CN^-), that previously have been shown to reduce intracellular ATP in isolated adult rat cardiomyocytes by 90% (Myrmel and Larsen, 1992). Hypoxia and glucose-free 30 mM K^+-HEPES buffer with or without CN^- had no effects on specific [^{3}H](+)-isradipine binding in cultured adult rat ventricular cardiomyocytes. High

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extracellular ATP inhibited binding in cardiomyocytes incubated in 30 mM or 120 mM K^+-HEPES buffer with physiological extracellular Ca^{2+} and Mg^{2+} concentrations. Our findings indicate that ATP has different effects on L-type Ca^{2+} channels in intact cardiomyocytes and in membranes.

2. Materials and methods

2.1. Materials

[5-methyl-^{3}H](+)-Isradipine (PN 200–110) was bought from Du Pont de Nemours, NEN Division, Dreieich, Germany. Nifedipine was a gift from Bayer, Leverkusen, Germany and (+)-isradipine from Sandoz, Basel, Switzerland. ATP (adenosine 5′-triphosphate disodium salt), essentially fatty acid-free bovine serum albumin, DL-carnitine, creatine, insulin, medium 199 with Earle's salts, 25 mM HEPES, bicarbonate and without glutamate, taurine, 3,3′,5-triiodo-D-thyronine (T_3) and trypsin were bought from Sigma Chemical, St. Louis, MO, USA. Collagenase type II and deoxyribonuclease type I (DNAse) were purchased from Worthington Biochemical Cooperation, Freehold, NJ, USA. Minimum essential medium (S-MEM) Joklik modification without $NaHCO_3$ (Joklik medium), natural mouse laminin and penicillin/streptomycin were bought from GIBCO BRL, Life Technologies, Gaithersburg, MD, USA. Creatine kinase assay was bought from Boehringer Mannheim, Biochemica, Mannheim, Germany, seronorm (animal source) from Nycomed, Oslo, Norway and Micro BCA protein assay reagent kit from Pierce, Rockford, IL, USA. Wistar rats were from Møllegaard, Skensved, Denmark.

2.2. Isolation of cardiomyocytes

Adult male Wistar rats of 200–370 g were anaesthetized with 1–2 ml 50 mg/ml pentobarbital i.p. The hearts were quickly removed, placed in ice-cold saline and the aorta cannulated and coupled to the perfusion apparatus. Two hearts were retrogradely perfused and later mechanically dissociated by collagenase dissolved in Joklik medium (37°C, all solutions gassed with 95% O_2/5% CO_2) by a procedure slightly modified from that earlier described (Stokke et al., 1996). Trypsin (61.2 U/ml) was added to solution A (Viko et al., 1995). Solution C was made as before from solution A without trypsin and with 0.5 mM Ca^{2+} and 1% bovine serum albumin. DNAse (0.02 mg/ml) was added to solution B used for mechanic tissue dissociation in order to make the cells less sticky. The final pellet of ventricular cardiomyocytes was resuspended in 15 ml culture medium (medium 199 with substituents, see below) with 6% bovine serum albumin and allowed to precipitate for 15 min, the supernatant was removed, the cells resuspended in 15 ml culture medium and examined microscopically using a haemocytometer.

2.3. Cardiomyocyte culture

Cardiomyocytes were cultured as described earlier (Ellingsen et al., 1993). Six-well Costar cell culture clusters (9.5 cm^2) were precoated at least 1 h before seeding with laminin (10 μg/ml) for cell attachment. Excess laminin was removed and 2 ml of $\sim 3.5 \times 10^4$ cells/ml culture medium (medium 199 with 0.2% bovine serum albumin, 2 mM DL-carnitine, 5 mM creatine, 5 mM taurine, 0.1 μM insulin, 0.1 nM T_3, 100 IU/ml penicillin and 100 μg/ml streptomycin) was plated in each well. The cardiomyocytes were incubated (37°C, air/5% CO_2) for 1–2 h, the culture medium aspirated to remove the $\sim$ 30% unattached cardiomyocytes and replaced by fresh culture medium. Cardiomyocytes used in experiments were grown for 48 h in culture.

2.4. Cardiomyocyte viability

Rod-shaped cardiomyocytes were defined as viable. The cell yield from two rat hearts was $\sim$ 7 mill of which 80% of total counted were rod-shaped. 80% (at least 150 cells/well were counted) of the cardiomyocytes were rod-shaped after 1–2 h in culture and 70% after 48 h in culture (at the end of the binding experiments). Activity of creatine kinase was determined in the buffer surrounding the cells and in cells that were scraped off and homogenized by sonication (L-Converter CL 1015, Sonifier B-12 power supply, Branson Sonic Power Company, Danbury, CT, USA) for 60 s placed on ice. The samples were centrifuged and the supernatants stored at −70°C. The samples were thawed placed on ice and kinetic activity of creatine kinase was determined by enzymatic process using a Cobas Bio (F. Hoffmann-La Roche & Co., Diagnostica, Basle, Switzerland) (Urdal et al., 1983). Cardiomyocytes with less than 10% loss of total creatine kinase content into the buffer were defined as of good quality.

2.5. Radioligand binding

Cardiomyocytes were incubated (air/5% CO_2) with [^{3}H](+)-isradipine in culture medium or HEPES buffer (0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 20 mM HEPES, 5.5 mM glucose, 4.12 mM $NaHCO_3$, 1 mM $CaCl_2$ and 0.8 mM $MgSO_4$) with either low K^+ (5.5 mM KCl and 137 mM NaCl), high K^+ (30 mM KCl and 112.2 mM NaCl) or very high K^+ (120 mM K^+ and 22.2 mM NaCl) of pH 7.4 at 37°C for 90 min. Specific binding was defined as the difference in binding in the absence and presence of 1 μM nifedipine. The cardiomyocytes of each well were scraped off and the content immediately filtered through Whatman GF/F filters, each well was washed with 5 ml ice-cold 50 mM Tris/HCl of pH 7.4 and the filters were washed subsequently with 10 ml of the same buffer. Free radioligand concentration was determined in aliquots of supernatants centrifuged for 2 min at 8400 × g at room tempera-

ture. Radioactivity was counted in a Beckman LS 5000CE liquid scintillation counter (counting efficiency of 38%).

2.6. Protein

Protein was determined in 50 mM Tris/HCl by the method of Lowry et al. (1951) using seronorm as standard, giving a protein content of 245 ± 13 μg ($n = 8$)/well. In some experiments, protein was determined by the Micro BCA (bicinchoninic acid) protein assay reagent kit in HEPES buffer using bovine serum albumin as standard (Sorensen and Brodbeck, 1986), yielding a protein content of 351 ± 40 μg ($n = 8$)/well.

2.7. Mg^{2+} depletion

After 24 h, the culture medium was replaced by 5.5 mM K^+-HEPES buffer of pH 7.4 with or without 0.8 mM $MgSO_4$ added. The cells were incubated (37°C, air/5% CO_2) for another 24 h during which samples were collected for measurements of total Mg^{2+}, total Ca^{2+} and creatine kinase activity. The cardiomyocytes were also examined microscopically.

2.8. Analysis of total intracellular Ca^{2+} and Mg^{2+}

Cardiomyocytes were washed once with 5.5 mM K^+-HEPES buffer (without Mg^{2+} or Ca^{2+} added) of pH 7.4, 37°C, 500 μl deionized H_2O was added to each well and cells were scraped off, each sample consisting of cells from 10 wells. Cell suspension kept on ice was sonicated for 120 s and stored at −20°C until used. Cell suspension was dried in Teflon tubes overnight at 90°C, cooled, weighted, 250 μl 65% HNO_3 (Scanpure) was added, the tube sealed and placed in an incubator at 70°C for 2 h, cooled and diluted with deionized H_2O to 4 ml. Total amounts of Ca^{2+} and Mg^{2+} were determined by inductively coupled plasma atomic emission spectrometry (Perkin Elmer ICP/5500) as previously described (Verburg, 1992).

2.9. Hypoxic incubation conditions

Cardiomyocytes were incubated at 37°C for 2 h in 30 mM K^+ glucose-free HEPES buffer of pH 7.4 with or without 1 mM CN^- and gassed with 95% N_2/5% CO_2. Such incubations have been shown to reduce intracellular ATP after 2 h by 90% in the absence of CN^- and by more than 90% in the presence of CN^- (Myrmel and Larsen, 1992). Controls were incubated for 2 h in 30 mM K^+ glucose-containing HEPES buffer and gassed with air/5% CO_2. [^{3}H](+)-Isradipine was added at the beginning or after 2 h hypoxic/normoxic incubation. When [^{3}H](+)-isradipine was added after 2 h, the incubations were prolonged with 60 min to allow binding. The cell viability was assessed by creatine kinase measurements after completed radioligand incubation.

2.10. Data analysis

Values given are mean ± S.E.M. (from at least three separate experiments). K_d and B_{max} were calculated according to Scatchard (Scatchard, 1949). Straight linear regression and the correlation coefficient were applied to assess linearity between variables. The significant difference of the mean was calculated using paired Student's t-test ($P < 0.05$, two-sided).

3. Results

3.1. Cell viability

Table 1 shows percentage rod-shaped cardiomyocytes and the percentage creatine kinase released into the culture medium during short-time culture of cardiomyocytes. Cultured cardiomyocytes used in this study had at least 70% rod-shaped appearance and less than 10% (in most cell batches less than 5%) of total creatine kinase activity of the cardiomyocytes had leaked into the buffer unless otherwise indicated, suggesting that the cells were viable and of good quality.

3.2. Specific binding of [^{3}H](+)-isradipine in cardiomyocytes

The level of specific binding was stable in cardiomyocytes up to 50 h in culture. The specific binding appeared to reach a peak after ~ 22 h; however, this increase was not statistically significant. All subsequent experiments were carried out on cardiomyocytes after 48 h in culture.

The binding of Ca^{2+} channel antagonists to cardiac L-type Ca^{2+} channels and the function of L-type Ca^{2+} channels vary with the membrane potential (Hess et al., 1984). We therefore investigated if specific [^{3}H](+)-isradipine binding increased with increasing extracellular K^+ concentrations. Specific binding of 0.24 nM [^{3}H](+)-isradipine in cardiomyocytes was increased 4-fold by increasing the extracellular K^+ concentration from 5.5 mM K^+ to ~ 30 mM K^+ (Fig. 1A). Binding-saturation experiments were performed to explain the effect of extracellular 5.5 and 30 mM K^+ on binding in cardiomyocytes (Fig. 1B). Scatchard plots of the binding-saturation data showed

Table 1
Cardiomyocyte viability with time in culture (h)

	1–2 h	18 h	42 h	72 h
% rod-shaped cells	80 ± 7	76 ± 9	69 ± 9	62 ± 11
% creatine kinase released	Not determined	5.6 ± 2.7	5.4 ± 2.5	3.9 ± 1.7

The percentage rod-shaped cardiomyocytes before seeding was 66 ± 9 and the batches contained (4.3 ± 0.6) × 10^4 cells/ml culture medium of 5.5 mM K^+, shown is the mean ± S.E.M. ($n = 3$ different cell batches).

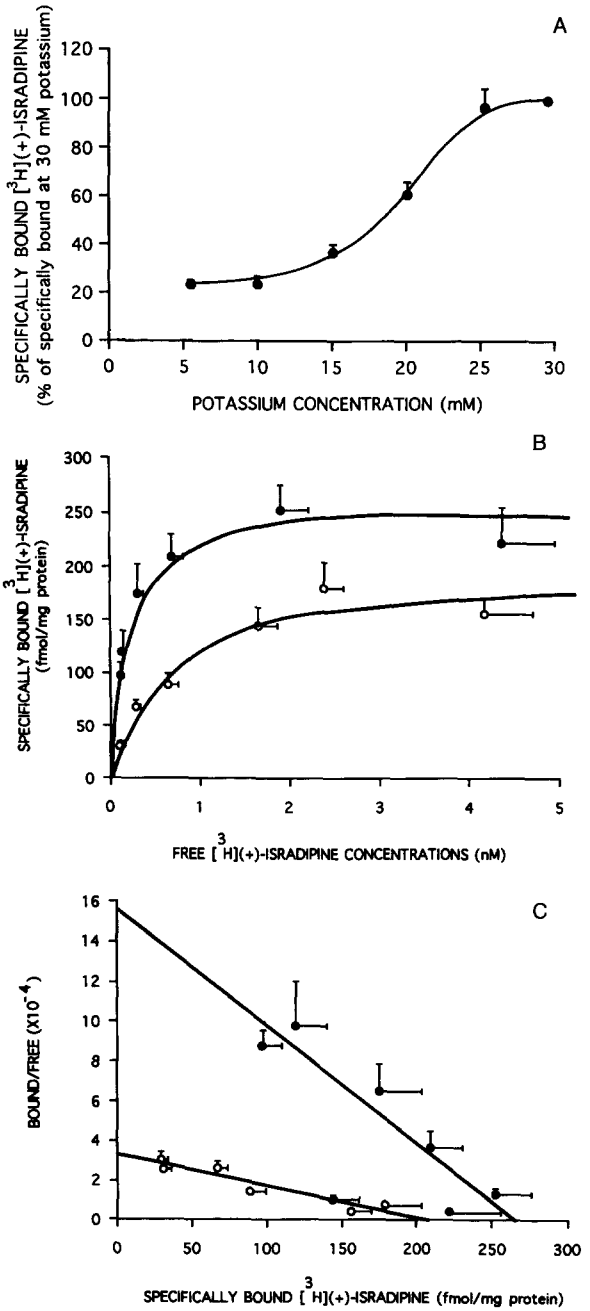

Fig. 1. Specific [^{3}H](+)-isradipine binding in cultured adult rat cardiomyocytes at 37°C. (A) Specific binding of 0.24 nM [^{3}H](+)-isradipine as a function of increasing K^+ concentration in culture medium (isoosmolar K^+ solution was added giving final K^+ concentrations from 5.5 mM to 29 mM) in 48 h cultured cardiomyocytes, mean ± S.E.M. ($n = 6$ experiments, each performed in duplicate). (B) Specific [^{3}H](+)-isradipine binding as a function of increasing concentration of [^{3}H](+)-isradipine in 48 h cultured cardiomyocytes, (○) incubated in 5.5 mM K^+-HEPES buffer and (●) in 30 mM K^+-HEPES buffer, mean ± S.E.M. ($n = 5$ experiments, each performed in triplicate). Curves between points were drawn according to the Scatchard equation. (C) Scatchard plots of binding-saturation experiments shown in (B), (○) in 5.5 mM K^+-HEPES buffer, $K_d = 0.62$ nM and $B_{max} = 204$ fmol/mg protein (from regression analysis, correlation coefficient of −0.94) and (●) in 30 mM K^+-HEPES buffer, $K_d = 0.17$ nM and $B_{max} = 264$ fmol/mg protein (from regression analysis, correlation coefficient of −0.94). The values of K_d and B_{max} were based on Scatchard analysis of data from five experiments.

that the mean K_d was 0.66 ± 0.07 nM in 5.5 mM extracellular K^+ and 0.22 ± 0.07 nM in 30 mM extracellular K^+, while the mean B_{max} was 224 ± 24 fmol/mg protein and 291 ± 28 fmol/mg protein, respectively (mean from Scatchard analysis of five individual Scatchard plots) (Fig. 1C). Thus, increasing the extracellular K^+ significantly decreased the K_d ($P < 0.02$) and hence increased the binding, while the tendency to increase the density of binding sites was not significant ($0.05 < P < 0.10$). Most of the experiments in this study were carried out at both 5.5 mM and 30 mM extracellular K^+ concentrations to mimic varying membrane potentials of cardiomyocytes in vivo (Wei et al., 1989).

3.3. Effect on binding of reducing intracellular Mg^{2+} concentration

We wanted to test if high concentrations of uncomplexed intracellular endogenous ATP could inhibit binding in cardiomyocytes. In order to increase uncomplexed endogenous ATP, the total intracellular Mg^{2+} concentration was reduced by 45%, from ~2.8 mM to 1.5 mM, by incubating cardiomyocytes for 24 h in Mg^{2+}-free HEPES buffer. Percentage creatine kinase released into the incubation buffer at the end of the binding experiment was close to 20%. Percentage rod-shaped cell was 70% after Mg^{2+}-reducing procedure and 50% at the end of binding experiments. These changes were found both in Mg^{2+}-reduced cells and controls, indicating that the Mg^{2+}-reducing procedure did not affect cell quality. Table 2 shows the effect of reducing total intracellular Mg^{2+} concentration on binding in 5.5 mM or 30 mM K^+-HEPES buffer without and with 0.8 mM Mg^{2+} added (allowing intracellular Mg^{2+} refilling (Quamme and Rabkin, 1990)) during binding incubation. We found no effect on [^{3}H](+)-isradipine binding in cardiomyocytes by reducing the total intracellular Mg^{2+} by 45%.

Table 2
Effects on 0.24 nM [^{3}H](+)-isradipine binding of reducing total intracellular Mg^{2+} by 45%

	Control cells	Mg^{2+}-depleted cells
In 5.5 mM K^+-HEPES buffer		
[^{3}H](+)-Isradipine incubation		
Without Mg^{2+} added	100	106 ± 18
With Mg^{2+} added (refilling)	99 ± 13	112 ± 22
In 30 mM K^+-HEPES buffer		
[^{3}H](+)-Isradipine incubation		
Without Mg^{2+} added	100	123 ± 17
With Mg^{2+} added (refilling)	86 ± 10	116 ± 11

Specific binding was divided on total creatine kinase activity/well, and calculated as percentage of controls in HEPES buffers without 0.8 mM Mg^{2+} during incubation, shown is the mean ± S.E.M. ($n = 3$ experiments, each performed with three and six parallels).

3.4. Effect on binding of hypoxia and glucose-free buffer with or without CN^-

High concentrations of ATP inhibited [^{3}H](+)-isradipine binding in rat heart membranes. In line with this, binding might be expected to be increased in cardiomyocytes with reduced intracellular ATP concentration. Cardiomyocytes were incubated using hypoxic protocols (2 h incubation in N_2 gas *and* glucose-free buffer with and without CN^-) previously shown to reduce intracellular ATP in isolated adult rat cardiomyocytes by 90% (Myrmel and Larsen, 1992). These incubations did not alter the percentage creatine kinase released from cardiomyocytes into the incubation buffer, suggesting that the cells were viable. Table 3 shows the effect of hypoxia and glucose-free 30 mM K^+-HEPES buffer with CN^- on 0.24 nM [^{3}H](+)-isradipine (adding [^{3}H](+)-isradipine at the beginning of the 2 h incubation) binding in cardiomyocytes compared with controls (normoxia). Hypoxia and glucose-free buffer with CN^- increased the specific 0.24 nM [^{3}H](+)-isradipine binding by 46%. Further, Table 3 shows the effect of hypoxia and glucose-free 30 mM K^+-HEPES buffer with or without CN^- on 1 nM [^{3}H](+)-isradipine binding compared with controls (normoxia). [^{3}H](+)-Isradipine was added at the beginning (group 1) or after 2 h hypoxic incubation (group 2). Hypoxia and glucose-free 30 mM K^+-HEPES buffer with or without CN^- had no significant effects on specific 1 nM [^{3}H](+)-isradipine binding in either group. Binding-saturation experiments were also carried out to test if hypoxia and glucose-free 30 mM K^+-HEPES buffer with CN^- affected [^{3}H](+)-isradipine (added at the start of the 2 h incubation) binding in cardiomyocytes. Scatchard analysis of binding-saturation data showed that the mean B_{max} was 264 ± 32 fmol/mg protein in hypoxic cells and 223 ± 15 fmol/mg protein in normoxic cells and the mean K_d was 0.15 ± 0.02 nM and 0.19 ± 0.03 nM, respectively ($n = 2$). Thus, hypoxia and glucose-free 30 mM K^+-HEPES buffer with CN^- had no effects on [^{3}H](+)-isradipine binding in cardiomyocytes. We also tested if 1 mM CN^- influenced binding in heart membranes. The specific [^{3}H](+)-isradipine binding in membranes was not affected by the presence of 1 mM CN^- (data not shown), suggesting that CN^- has no direct effects on L-type Ca^{2+} channels. Taken together, most of our experiments showed that long-lasting hypoxia and glucose-free 30 mM K^+-HEPES buffer with and without CN^- have no effect on specific [^{3}H](+)-isradipine binding to L-type Ca^{2+} channels in cardiomyocytes.

Table 3
Effects of 2 h hypoxia and glucose-free 30 mM K^+-HEPES buffer with or without CN^- on [^{3}H](+)-isradipine binding

Concentration of [^{3}H](+)-isradipine added	Normoxia	Hypoxia	Hypoxia and CN^-
Group 1			
0.24 nM	100		146 ± 8 [a]
1 nM	100	90 ± 9	117 ± 23
Group 2			
1 nM	100	82 ± 21	127 ± 27

Specific binding was calculated as percentage of controls (normoxia) in each group, mean ± S.E.M. ($n = 4$ experiments for each concentration of [^{3}H](+)-isradipine, each performed with six or nine parallels). The specific binding of controls (normoxia) in group 1 and in group 2 did not differ significantly, [a] $P < 0.02$, compared with its own control (normoxia).

Table 4
Effects of extracellular 1 mM ATP, 1 mM Ca^{2+} and 0.8 mM Mg^{2+} on 0.24 nM [^{3}H](+)-isradipine binding

	Control	1 mM ATP
In 5.5 mM K^+-HEPES buffer		
With Ca^{2+} and Mg^{2+}	100	89 ± 8 ($n = 7$)
Without Ca^{2+} and Mg^{2+}	88 ± 2 [a] ($n = 4$)	131 ± 19 ($n = 4$)
In 30 mM K^+-223HEPES buffer		
With Ca^{2+} and Mg^{2+}	100	97 ± 4 ($n = 7$)
Without Ca^{2+} and Mg^{2+}	116 ± 4 [a] ($n = 4$)	120 ± 4 [a] ($n = 4$)

The effects were calculated as percentage of controls in HEPES buffers with Ca^{2+} and Mg^{2+} without ATP added. Shown is the mean ± S.E.M. from the number of experiments indicated in brackets, each performed with three parallels in the presence of ATP and six parallels in the controls. [a] $P < 0.05$.

3.5. Effects of extracellular ATP on binding

We tested if high extracellular ATP concentration inhibited [^{3}H](+)-isradipine binding in cardiomyocytes. Table 4 shows that extracellular 1 mM ATP had no effects on [^{3}H](+)-isradipine binding in cardiomyocytes incubated in 5.5 mM or 30 mM K^+-HEPES buffer with physiological Ca^{2+} and Mg^{2+} concentrations.

We investigated if 1 mM ATP had effect on binding when Ca^{2+} and Mg^{2+} were excluded from the HEPES buffers. Table 4 shows that excluding Ca^{2+} and Mg^{2+} decreased the binding in cardiomyocytes by 12% in 5.5 mM K^+-HEPES buffer, whereas the binding in cardiomyocytes increased by 16% in 30 mM K^+-HEPES buffer. Extracellular 1 mM ATP increased specific binding of [^{3}H](+)-isradipine by 20% in 30 mM K^+-HEPES buffer without Ca^{2+} and Mg^{2+}; however, this effect was not significantly different from that produced alone by omitting Ca^{2+} and Mg^{2+} from the buffer. This indicates that it was the exclusion of Ca^{2+} and Mg^{2+} that increased the binding and not ATP. Thus, extracellular 1 mM ATP had no significant effect on binding in 5.5 mM or 30 mM K^+-HEPES buffer without Ca^{2+} and Mg^{2+}. Excluding divalent cations from the HEPES buffers significantly decreased the binding in cardiomyocytes in 5.5 mM K^+ buffer and significantly increased the binding in cardiomyocytes in 30 mM K^+ buffer.

We also examined the effect of a wider concentration range of extracellular ATP on binding in cardiomyocytes

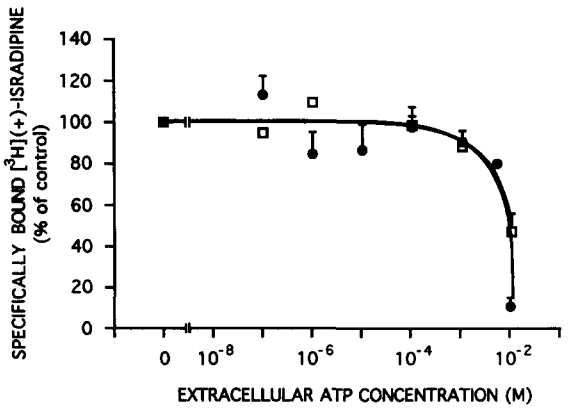

Fig. 2. Effect of increasing extracellular ATP concentration on specific 0.24 nM [^{3}H](+)-isradipine binding in ● 30 mM or □ 120 M K^+-HEPES buffer at 37°C in 48 h cultured cardiomyocytes, mean ± S.E.M. ($n = 2$ experiments for each buffer, each performed with six parallels).

in 30 mM or 120 mM K^+-HEPES buffer with Ca^{2+} and Mg^{2+} (Fig. 2). Extracellular 10 mM ATP inhibited 0.24 nM [^{3}H](+)-isradipine binding by 90% in 30 mM K^+-HEPES buffer and by 50% in 120 mM K^+-HEPES buffer. Thus, extracellular ATP concentrations less or equal to 1 mM ATP had no effect on [^{3}H](+)-isradipine binding in cardiomyocytes, while higher concentrations inhibited the binding in 30 mM and 120 mM K^+-HEPES buffer with Ca^{2+} and Mg^{2+}.

4. Discussion

The cardiomyocytes used in the present study were rod-shaped in culture and released small amounts of creatine kinase to the medium, indicating good cell viability. As in earlier studies, increasing extracellular K^+ from 5.5 mM to 30 mM decreased K_d of specific [^{3}H](+)-isradipine binding in cardiomyocytes without significant effects on B_{max} (Kokubun et al., 1986; Wei et al., 1989). A high extracellular K^+ concentration has, however, also been reported to increase B_{max} without effects on K_d in cardiomyocytes (Green et al., 1985; Lee et al., 1987).

The aim was to test if ATP affected specific [^{3}H](+)-isradipine binding to L-type Ca^{2+} channels in cardiomyocytes by acting from the intracellular or the extracellular side of the sarcolemma.

Two distinct effects of intracellular high concentration of ATP might be hypothesized: a protective effect on L-type Ca^{2+} channels, preventing channel run-down observed in single ventricular cardiomyocytes (Sperelakis and Schneider, 1976; Belles et al., 1988; Ono and Fozzard, 1992); and a direct, reversible inhibitory effect on specific [^{3}H](+)-isradipine binding to L-type Ca^{2+} channels observed in membranes (Glossmann et al., 1982; Stokke et al., 1996). We tested if depletion of intracellular ATP affected binding. No *consistent* change in specific [^{3}H](+)-isradipine binding was caused by long-lasting hypoxia and glucose-free buffer with and without CN^-. In particular, we found no reduction in binding and, thus, no support for the view that loss of ATP leads to channel destruction. In some experiments, binding even showed a tendency to increase which could indicate that an inhibitory effect of ATP on binding was removed. The results were similar whether [^{3}H](+)-isradipine was added at the beginning of the hypoxic period or after a period of 2 h. This finding excludes that presence of [^{3}H](+)-isradipine prevents changes in L-type Ca^{2+} channels caused by hypoxia in cardiomyocytes as shown in membranes from ischemic tissue (Van Amsterdam et al., 1990). Our results show for the first time that even profound lowering of ATP is not enough to reduce specific [^{3}H](+)-isradipine binding in intact, cultured cardiomyocytes. Likewise, Gu et al. (1989) have reported no effect on 1,4-dihydropyridine antagonist binding in membranes from isolated rat hearts exposed to 30 or 60 min hypoxia (hearts perfused with buffer gassed with N_2). In contrast, the binding density of 1,4-dihydropyridine antagonists has been shown to be reduced in membranes and homogenates prepared from 30–60 min global ex vivo ischemic hearts (Gu et al., 1988) and from in vivo porcine ischemic heart regions (Kirkebøen et al., 1995). We can only speculate on reasons for this difference. The binding density of 1,4-dihydropyridine antagonists to L-type Ca^{2+} channels might be reduced in membranes and homogenates from ischemic tissue due to activation of enzymes like proteases, lipases and phosphatases (Jennings and Reimer, 1991), destroying L-type Ca^{2+} channels before or during tissue preparation (Yoshida et al., 1992; De Jongh et al., 1994).

We tested if extracellular ATP caused direct inhibition of specific [^{3}H](+)-isradipine binding. Previously, we have found that ATP inhibited specific 0.12 nM [^{3}H](+)-isradipine binding in membranes at low Ca^{2+} and Mg^{2+} concentrations with a slope factor of one and with 50% inhibition at 0.4 mM ATP. Like in membranes and homogenates, ATP applied extracellularly caused significant inhibition of specific [^{3}H](+)-isradipine binding in cardiomyocytes in both 30 mM and 120 mM K^+-HEPES buffer (depolarizing buffer). However, ATP was far less potent in inhibiting [^{3}H](+)-isradipine binding in cells than in membranes.

Intact cells and isolated membranes also behaved differently with respect to Ca^{2+} and Mg^{2+}. Specific [^{3}H](+)-isradipine binding in cardiomyocytes was only slightly affected by the presence of extracellular Ca^{2+} and Mg^{2+}. Kokubun et al. (1986), too, found little effect of extracellular Ca^{2+} on 1,4-dihydropyridine antagonist binding in cardiomyocytes, while others found that increasing extracellular Ca^{2+} increased binding in 137 mM K^+ buffer (Porzig and Becker, 1988). Increasing Ca^{2+} and Mg^{2+} concentrations are known to reversibly increase 1,4-dihydropyridine antagonist binding to L-type Ca^{2+} channels in membranes (Glossmann et al., 1982; Glossmann and Striessnig, 1988). In line with this, we examined if a

moderate reduction in total intracellular Mg^{2+} altered specific [^{3}H](+)-isradipine binding by increasing the inhibitory effect of ATP on binding. However, reducing the total intracellular Mg^{2+} by 45% did not alter specific [^{3}H](+)-isradipine binding.

In conclusion, experiments with our hypoxic cardiomyocyte model provide evidence that even severe intracellular ATP depletion, associated with L-type Ca^{2+} channel run-down, does not cause reduction in binding density of the 1,4-dihydropyrine antagonist, [^{3}H](+)-isradipine, to L-type Ca^{2+} channels. Thus, 1,4-dihydropyridine antagonist binding to L-type Ca^{2+} channels is differently modulated by long-lasting hypoxia in intact cardiomyocytes in culture and by myocardial ischemia followed by tissue preparation. Also, the direct inhibitory effect of extracellular ATP on 1,4-dihydropyrine antagonist binding to L-type Ca^{2+} channels in cardiomyocytes differs from the ATP effect on binding in isolated membranes. Apparently, care must be taken when extending results of binding studies with radiolabeled Ca^{2+} channel antagonists from membranes to intact cells.

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