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Cytosolic Ca^{2+} domain-dependent protective action of adenosine in cardiomyocytes

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Abstract

Recently, in beating cardiac cells heterogeneous spatiotemporal patterns in cytosolic Ca^{2+} distribution have been visualized, and associated with cell contraction. In non-beating cardiomyocytes, spatial heterogeneity of intracellular Ca^{2+} distribution has also been observed, yet its functional implication in resting cardiac cells is not known. Herein, distinct domains of lower versus higher concentrations of cytosolic Ca^{2+} (0.17 and 0.37 μM, respectively) were observed using epifluorescent digital imaging in single, non-beating, fluo-3-loaded cardiomyocytes. Extracellular K^+ (16 mM) induced a uniform increase of cytosolic Ca^{2+}, despite the initial presence of distinct domains of cytosolic Ca^{2+} (from 0.17 to 1.82 μM in domains with lower, and from 0.37 to 2.03 μM in domains with higher Ca^{2+} concentration, respectively). In contrast, adenosine (1 mM) prevented extracellular K^+ to induce cytosolic Ca^{2+} loading selectively within domains with lower (from 0.17 to 0.18 μM), but not in domains with higher (from 0.37 to 1.4 μM) basal Ca^{2+} concentration. Thus, the response of a cardiomyocyte to the protective action of adenosine is heterogeneous within a resting single cell. The domain-distinct cytoprotective action of adenosine appears to be set by the basal Ca^{2+} concentration within a cytosolic domain.

Keywords: Adenosine; Ca^{2+}, cytosolic; Ca^{2+} domain; Cardioprotection; Fluo-3; Heart

1. Introduction

Imaging techniques have revealed that the distribution of Ca^{2+} within the cytosol of various mammalian and non-mammalian cells at rest, such as epithelial cells, neurons or smooth muscle cells can be non-homogenous (Lipscombe et al., 1988; Neher and Augustine, 1992; Clapham, 1995). Factors responsible for the spatial variations of Ca^{2+} concentration in these cells include receptor-mediated Ca^{2+} mobilization, morphological polarity or functional stages of the cell cycle such as methaphase to anaphase transition (Speksnijder, 1992; Lischka and Schild, 1993; Stendahl et al., 1994; Gu et al., 1994; O'Malley, 1994; Eilers et al., 1995). Non-homogenous distribution of Ca^{2+} has been associated with differential regulation of cellular processes as diverse as development and plasticity of the nervous system, modulation of secretion or cell death (Ghosh and Greenberg, 1995).

In cardiac cells, the concentration of Ca^{2+} during diastole is low (100–200 nM) and usually rather homogenous (Wier et al., 1987). Although spatial heterogeneity of intracellular Ca^{2+} concentration has been demonstrated during contractile activity, it has also been observed at rest (Williford et al., 1990; Cheng et al., 1993; Williams, 1993; Niggli and Lipp, 1995). While dynamic, oscillatory, changes in Ca^{2+} concentration after depolarization have been associated with cell contraction (for review see Niggli and Lipp, 1995), the functional implication of Ca^{2+} domains in non-beating cardiomyocytes is not known.

It is established that elevation of the concentration of extracellular K^+, that occurs during hyperkalemic challenges such as ischemia or cardioplegia, induces Ca^{2+} loading in myocardial cells which is detrimental for normal cardiac cell function (Powell et al., 1984; Cyran et al., 1993). Adenosine has been shown to prevent Ca^{2+} loading evoked by different insults (Touraki and Lazou, 1992; Fralix et al., 1993; Suleiman and Ashraf, 1995), and to possess cytoprotective properties during ischemia and cardioplegia (Ely et al., 1985; Liu et al., 1991; Auchampach and Gross, 1993; Vinten-Johansen et al., 1993). However, it is unknown whether spatial heterogeneity in the distribution of intracellular Ca^{2+} in single cells has some implications on the K^+-induced Ca^{2+} loading, and/or the cytoprotective action of adenosine.

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In the present study, we visualized a small percentage of non-beating cardiac cells with different domains of cytosolic Ca^{2+} concentration. To examine whether the existence of cytosolic Ca^{2+} domains has functional consequence(s), we investigated the cytoprotective action of adenosine against K^+-induced Ca^{2+} loading in this population of cardiac cells.

2. Materials and methods

2.1. Cells

Ventricular myocytes were isolated by enzymatic dissociation (Terzic et al., 1994). Guinea-pigs were anesthetized with pentobarbital and artificially ventilated. Aorta cannulated, and following cardiotomy the heart retrogradely perfused (at 37°C) with the following sequence of solutions (for composition, see below): first, control bathing solution for 5–10 min, then nominally Ca^{2+}-free bathing solution, followed by nominally Ca^{2+}-free solution containing collagenase (0.04 g per 100 ml, Sigma type IA with a purity that ranged from 94 to 96% and a tryptic activity of 0.63 units/mg solid) for 30 min, and finally high-K^+/low-Cl^- solution for 5 min. The heart was then stored in the high-K^+/low-Cl^- solution at 4°C. Single cells were isolated by agitating a small piece of dissected ventricle in a culture dish filled with the control bathing solution.

2.2. Intracellular loading with fluorescent probes

Cardiac cells were loaded with 3.5 μM of the Ca^{2+}-selective fluorescent probe, fluo-3 acetoxymethyl ester (fluo-3AM), for 20 min at room temperature (21–23°C). The loading conditions were selected to permit complete cleavage of the dye by intracellular esterases (Poenie et al., 1986; Minta et al., 1989; Kao et al., 1989). This probe exhibits lower binding capacity for Ca^{2+} and produces larger fluorescence signal following Ca^{2+} binding than conventional fluorescent probes (Eberhard and Erne, 1989; Minta et al., 1989; Niggli and Lederer, 1990). Myocytes, loaded with fluo-3AM, were transferred to an experimental chamber mounted on the stage of the epifluorescent microscope, and permitted to adhere to the glass bottom of the chamber. In a separate set of experiments cardiomyocytes were loaded with the ratiometric dye fura-2 acetoxylmethylester (fura-2AM; 10 μM) to quantify resting intracellular Ca^{2+} concentration.

2.3. Epifluorescent digital microscopy

Ventricular myocytes were imaged, at 21–23°C, by digital epifluorescent microscopy using an inverted microscope (Zeiss Axiovert-135 TV) with an 40 × oil-immersion objective lens. Optimal focus was adjusted by viewing myocytes under bright field microscopy. A 100 W mercury lamp served as a source of light to excite fluo-3AM at 488 nm (or fura-2AM at 340 and 380 nm). Fluorescence emitted at 520 nm by the 'excited' dyes was captured, after crossing a dichroic mirror, by an intensified charge coupled device camera (ICCD), and digitized using the epifluorescent imaging system (Attoflor RatioVision). Background fluorescence (Tyrode solution containing no cells) was subtracted from the fluorescence of fluo-3AM (or fura-2AM)-loaded myocytes.

2.4. Calibration of fura-2 and fluo-3 signals

In cells loaded with fura-2AM, an estimate of the Ca^{2+} concentration ($[Ca^{2+}]$) was obtained according to the equation:

$$[Ca^{2+}] = (R - R_{min})/(R_{max} - R) \cdot K_d \cdot \beta$$

where R is the fluorescence ratio recorded from the cell; R_{min} and R_{max} the fluorescence ratio when extracellular Ca^{2+} was removed (and 3 mM EGTA added to the extracellular solution) and at 3 mM $CaCl_2$, respectively; K_d the Ca^{2+} dissociation constant of the dye (236 nM); and β the ratio of R_{min}/R_{max} at 380 nm (Grynkiewicz et al., 1985; Williams, 1990). To carry out the calibration in situ, once the experimental data were collected in intact cardiomyocytes, fura-2AM-loaded cardiac cells where exposed to the Ca^{2+} ionophore, 4-bromo A-23187, and R_{min} and R_{max} were measured in such permealized cells. To prevent cell contraction in permealized cells exposed to high concentrations of extracellular Ca^{2+}, myocytes were pretreated with carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (2 μM) and 2,3-butanedione monoxime (40 mM).

An estimate of the increase in $[Ca^{2+}]_i$ as a function of fluo-3 fluorescence was calculated from the equation described by Xu and Tashjian (1995):

$$[Ca^{2+}] = K_d (F - F_{min})/(F_{max} - F) + [Ca^{2+}]_r$$

where F was the observed fluorescence, F_{min} was the fluorescence in the absence of Ca^{2+} (extracellular Ca^{2+} was removed and 3 mM EGTA added to the extracellular solution) and F_{max} was the fluorescence in the presence of saturating $[Ca^{2+}]$ (3 mM $CaCl_2$). To obtain F_{min} and F_{max} cells were exposed to the Ca^{2+} ionophore, A-23187, after the experiments were completed (see above). $[Ca^{2+}]_r$ is the resting $[Ca^{2+}]_i$ concentration determined in fura-2-loaded ventricular myocytes (see above), and K_d the Ca^{2+} dissociation constant of fluo-3 (422 nM) (Lipp and Niggli, 1993).

2.5. Experimental procedure

Rod-shaped, single cardiomyocytes with clear striation, but with cytosolic non-homogenous distribution of the fluo-3 fluorescence intensity were selected at the begin-

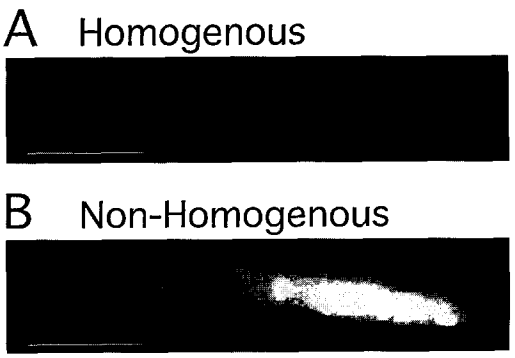

Fig. 1. Guinea-pig ventricular myocytes with homogenous (A) and non-homogenous (B) distribution of cytosolic Ca^{2+}. Horizontal bars indicate 20 μm.

ning of each experiment (Fig. 1B). To simplify data interpretation, only cells with two distinct regions of fluorescence were analyzed (see Fig. 1B). Within the 189 cardiomyocytes that were imaged, ~ 8% manifested these criteria. Myocytes were exposed first to Tyrode solution (control), then, adenosine (1 mM) was added, followed by adenosine (1 mM) plus 16 mM K^+, and finally, 16 mM K^+. Accordingly, each cell was its own control, and experiments in which cells did not respond to the elevation of extracellular K^+ were excluded from the analysis.

2.6. Drugs and solutions

The Tyrode solution contained (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, Hepes-NaOH 5.5 (pH 7.4). The composition of the high-K^+/low-Cl^- solution was (in mM): taurine 10, oxalic acid 10, glutamic acid 70, KCl 25, KH_2PO_4 10, glucose 11, EGTA 0.5, and Hepes-KOH 10 (pH 7.3–7.4). Adenosine (Sigma) was dissolved in Tyrode solution and diluted prior to the experiment. Fluo-3AM (Molecular Probes) was dissolved in dimethyl sulfoxide and pluronic acid, and fura-2 (Molecular Probes) in dimethyl sulfoxide. The final concentrations of dimethyl sulfoxide and pluronic acid in the bath did not exceed 0.1% and 0.004%, respectively.

2.7. Statistics

Results are expressed as mean ± S.E.M.; n refers to the number of experiments. Statistical significance was determined with the Student's t-test for paired observations, and a value of $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Distinct domains of cytosolic Ca^{2+} concentration in resting single cardiomyocytes

In the selected myocytes (see Materials and methods), two domains with lower versus higher Ca^{2+} concentrations were distinguished (Fig. 1B and Fig. 2A). In average, the Ca^{2+} concentration was 0.17 ± 0.01 μM and 0.37 ± 0.02 μM in domains with lower and higher Ca^{2+} concentration, respectively ($P < 0.01$; $n = 4$; see also first bar pairs in Fig. 2B).

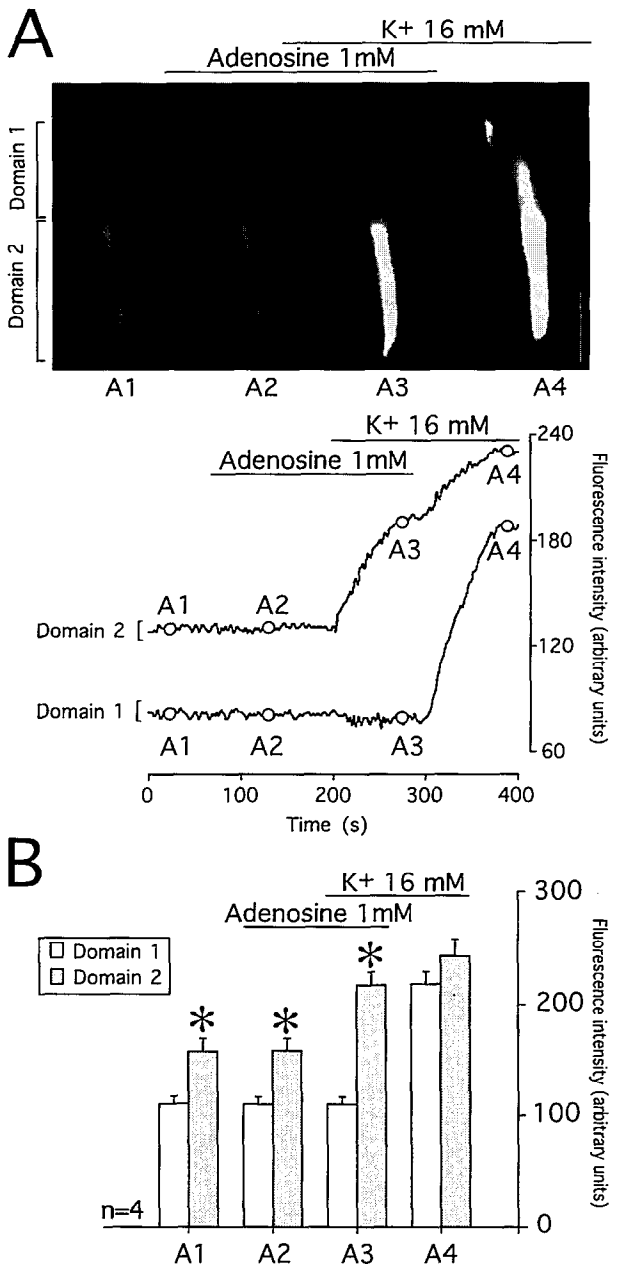

Fig. 2. Domain-restricted protection by adenosine against K^+-induced Ca^{2+} loading. A: Epifluorescent frames from a cardiomyocyte (upper panel), and corresponding changes in intensity of fluo-3 fluorescence (lower panel). Vertical bar indicates 20 μm. Open circles in lower panel correspond to frames in upper panel. Changes in fluorescence in domains with lower (domain 1) and higher (domain 2) basal cytosolic Ca^{2+} concentration in Tyrode (A_1), in adenosine (A_2), in adenosine plus 16 mM K^+ (A_3), and in plus 16 mM K^+ (A_4). B: Average changes in maximal fluorescence under conditions A_{1-4}. Each column represents mean ± S.E.M. Asterisks indicate significant difference between domains.

3.2. Extracellular K^+-induced homogenous Ca^{2+} loading

Elevation of extracellular K^+ to 16 mM induced an increase in intracellular Ca^{2+} in cardiomyocytes (Fig. $2A_4$). This increase was uniform throughout a cells despite the pre-existence of different resting concentrations of Ca^{2+} within cytosolic domains (Fig. $2A_4$). Following elevation of extracellular K^+, cytosolic Ca^{2+} concentration reached 1.82 ± 0.19 μM in domains with lower resting concentrations of Ca^{2+} ($n = 4$; Fig. $2A_4$), and 2.03 ± 0.26 μM ($n = 4$) in domains with higher resting Ca^{2+} (Fig. $2A_4$). When compared, the values after the K^+ challenge were not significantly different between different domains ($P > 0.05$; Fig. 2B).

3.3. Lack of effect of adenosine on cytosolic Ca^{2+} domains in resting cardiomyocytes

Exposure of cardiomyocytes to adenosine (1 mM) did not affect Ca^{2+} concentration either in domains with low or in domains with high resting Ca^{2+} concentration (Fig. $2A_1$–A_2). In average, the estimated Ca^{2+} concentration in domains with lower resting Ca^{2+} was 0.17 ± 0.01 μM prior, and remained 0.17 ± 0.01 μM after addition of adenosine ($n = 4$, $P > 0.05$; Fig. 2B). Also, in domains with higher resting Ca^{2+} concentration the estimated Ca^{2+} concentration was 0.37 ± 0.02 μM prior, and remained 0.37 ± 0.02 μM, after addition of adenosine ($n = 4$, $P > 0.05$; Fig. 2B).

3.4. Domain-dependent effect of adenosine on K^+-induced Ca^{2+} loading

Adenosine (1 mM) prevented K^+-induced Ca^{2+} loading in domains with lower basal Ca^{2+} concentration within cardiomyocytes (Fig. $2A_3$). In the presence of adenosine, addition of 16 mM of extracellular K^+ increased the apparent average Ca^{2+} concentration to 0.18 ± 0.01 μM ($n = 4$). This value is not significantly different from the value obtained in domains with lower basal Ca^{2+} concentration under control conditions ($n = 4$; $P > 0.05$; Fig. 2B). By contrast, in domains with higher basal cytosolic Ca^{2+}, adenosine (1 mM) did not prevent 16 mM extracellular K^+ to induce a significant elevation in Ca^{2+} (Fig. $2A_3$). In these domains, Ca^{2+} concentration increased to 1.4 ± 0.15 μM ($n = 4$). This value is significantly different from the value obtained in domains with higher basal Ca^{2+} concentration under control conditions ($n = 4$; $P < 0.01$; Fig. 2B).

4. Discussion

Diverse spatiotemporal patterns of cytosolic Ca^{2+} distribution, ranging from Ca^{2+} microdomains to Ca^{2+} waves, have been recently visualized by imaging techniques in non-mammalian and mammalian cells (Linas et al., 1992; Berridge, 1993; Clapham, 1995). In the present study, we also observed a non-homogenous distribution of cytosolic Ca^{2+} in a small percentage of resting guinea-pig ventricular cells.

In principle, the apparent existence of domains with different Ca^{2+} concentrations can be genuine in nature or can be due to an artifact such as an uneven accumulation or exclusion of the Ca^{2+} fluorescent probe from subcellular organelles (Silver et al., 1990). This later possibility seems unlikely since the fluorescent probe fluo-3, used herein, has a minimal tendency for accumulation, and/or active extrusion from subcellular organelles (Kao et al., 1989; Niggli and Lederer, 1990). Also, the manifestation of apparent Ca^{2+} gradients could be due to a different sensitivity of the Ca^{2+} fluorescent dye to various other ions, in particular H^+, which on their own can distribute unevenly. However, contrary to its congeners, fluo-3 is highly selective for Ca^{2+}, and the intensity of fluo-3 fluorescence is not affected by changes in intracellular pH (Minta et al., 1989). It should also be pointed out that apparent differences in basal cytosolic fluorescence, visualized by digital epifluorescent imaging, may be due to the contribution of out-of-focus fluorescence. Using laser confocal microscopy, which effectively removes out-of-focus fluorescence and minimizes artifactual contributions due to changes in cell shape or volume, we (unpublished results) and other (Williams, 1990, 1993; Williams et al., 1992) have also visualized heterogeneous distribution in cytosolic Ca^{2+} in cardiac cells. Taken together, these findings suggest that regional differences in fluo-3 fluorescence, observed herein, are genuine, and apparently not the consequence of an artifact.

In general, the distribution of intracellular Ca^{2+} in non-beating heart cells has been described as homogenous (Wier et al., 1987; Cheung et al., 1989). However, different heterogeneous spatiotemporal patterns of intracellular Ca^{2+} distribution have also been demonstrated, albeit with a low probability of occurrence in the mammalian myocardium at diastolic levels of Ca^{2+} (Lakatta et al., 1985; Williford et al., 1990; Ishide et al., 1990; Williams et al., 1992; Williams, 1993). Our results are in accord with these findings since in the majority of visualized cells we did not observe distinct domains of Ca^{2+} concentration (Fig. 1A), and non-homogeneous patterns occurred in a small percentage of resting myocytes.

The variation of the estimated Ca^{2+} concentration between cellular domains varied between the lower and upper limits for the Ca^{2+} concentration previously reported in cardiomyocytes at rest (Marban et al., 1980; Wier et al., 1987). The origin of the heterogeneity in intracellular Ca^{2+} distribution is not yet known. Cytosolic Ca^{2+} is regulated by Ca^{2+} transport mechanisms located in the sarcolemma and membranes of intracellular Ca^{2+} stores, as well as by the Ca^{2+} buffering capacity of the cytosol determined by the affinities for Ca^{2+} of various

Ca^{2+}-binding proteins (Carafoli, 1994; Roberts, 1994). Selective expression of Ca^{2+} conductive pathways within restricted cellular domains, and 'clustering' of Ca^{2+} channels has been previously proposed to be associated with the existence of cytosolic Ca^{2+} gradients in non-cardiac tissue (Lischka and Schild, 1993). It is conceivable that the existence of Ca^{2+} gradients in cardiomyocytes also reflects differences in the anatomical distribution or efficiency of Ca^{2+} handling mechanisms.

It is known that elevation of K^+ concentration in the extracellular milieu induces depolarization of cardiac cells and consequently Ca^{2+} loading (Powell et al., 1984; Cyran et al., 1993). The present study confirms this effect of K^+, and further demonstrates that the elevation of Ca^{2+} induced by extracellular K^+ is homogenous, at least at this level of spatial resolution. The homogenous K^+-induced Ca^{2+} loading occurred even in cells expressing heterogeneous domains of cytosolic Ca^{2+} concentrations. Accordingly, the existence of heterogeneous Ca^{2+} domains has, apparently, no consequence on the response of the cell to K^+-induced Ca^{2+} loading. Recently, it has been shown that the extent of increase in intracellular Ca^{2+} concentration in electrically stimulated heart cell populations, with different basal Ca^{2+} concentration, also does not depend on the resting levels of intracellular Ca^{2+} (Ahlemeyer et al., 1993). Our findings would be in accord with the concept that heterogeneous basal Ca^{2+} concentration present within a single cell (present study) or in different cell populations (Ahlemeyer et al., 1993) does not affect the magnitude of Ca^{2+} loading induced by chemical or electrical membrane depolarization.

We report that adenosine differentially prevented, within myocytes, extracellular K^+ to induce cytosolic Ca^{2+} loading in domains with lower, in contrast to domains with higher basal Ca^{2+} concentration. Previously, populations of cardiomyocytes with significantly different resting Ca^{2+} concentrations have been isolated from the same heart (Hayashi et al., 1990; Ahlemeyer et al., 1992a, b). These cell populations, with different resting Ca^{2+} concentration, displayed different responses to metabolic challenges or to cardiac glycosides (Hayashi et al., 1990; Ahlemeyer et al., 1992a, b). The present finding that adenosine prevents K^+-induced Ca^{2+} loading preferentially in domains with low basal Ca^{2+} concentration is in agreement with the notion that levels of resting Ca^{2+} can set the sensitivity and/or responsiveness of a cardiomyocyte toward drug action. It appears that such principle may apply not only to cell populations with different resting Ca^{2+} concentrations, but also to single cells with different Ca^{2+} concentrations within cytosolic domains.

Although the property of adenosine to protect the myocardium against various insults has been recognized, the cardioprotective efficacy of adenosine is variable (Ely et al., 1985; Liu et al., 1991; Auchampach and Gross, 1993; Vinten-Johansen et al., 1993; Genote et al., 1993). The ability of a cardiac cell to differentially respond to adenosine (associated, in the present study, with the existence of heterogeneous regions of Ca^{2+} concentrations) may contribute to the non-uniform nature of the cardioprotective action of adenosine. The underlying mechanism of the domain-restricted protective action of adenosine is at present unknown. The cytoprotective action of adenosine has been previously associated to the activation of ATP-sensitive K^+ channels (Grover et al., 1992; Auchampach and Gross, 1993; Offstad et al., 1994) and/or protein kinase C (Armstrong et al., 1994; Baxter et al., 1995). It is appealing to speculate that the domain-restricted action of adenosine may be related to the differential Ca^{2+}-mediated regulation of ATP-sensitive K^+ channels (Findlay, 1988) and/or protein kinase C (Mochly-Rosen, 1995) between domains with higher versus lower Ca^{2+} concentration. Further investigation is required to establish the mechanism responsible for the Ca^{2+} domain-dependent protective action of adenosine within single cardiomyocytes.

The presented results can be interpreted to indicate that (1) the protective action of adenosine against K^+-induced Ca^{2+} loading is not uniform throughout a cardiomyocyte, rather subcellular regions appear to be differentially protected, and that (2) the domain-restricted nature of adenosine-mediated cytoprotection is related to the existence of cytosolic domains with different Ca^{2+} concentration. Besides cardiac cells, a nonhomogeneous distribution of cytosolic Ca^{2+} has been reported in several cell types (Lipscombe et al., 1988; Neher and Augustine, 1992; Speksnijder, 1992; Lischka and Schild, 1993; Stendahl et al., 1994; Gu et al., 1994; O'Malley, 1994; Eilers et al., 1995), raising the possibility of a non-uniform ('Ca^{2+} domain-dependent') responsiveness to an agent within a single cell (see also Ghosh and Greenberg, 1995). Such a principle could suggest the functional implication of non-homogenous cytosolic Ca^{2+} distribution as a subcellular regulator of the action of a neurohormone or drug.

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