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Short communication

Dual effect of glyburide, an antagonist of K_{ATP} channels, on metabolic inhibition-induced Ca^{2+} loading in cardiomyocytes

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Abstract

Whether sulfonylurea therapy, which blocks ATP-sensitive K^+ (K_{ATP}) channels, impedes endogenous cardioprotective mechanisms during cellular metabolic impairment remains controversial. Therefore, the effect of glyburide, a prototype sulphonylurea drug, on cytosolic Ca^{2+} concentration and K_{ATP} channel activity, was measured in 2-4-dinitrophenol-treated guinea-pig cardiomyocytes, using epifluorescent digital-imaging and cell-attached patch-clamp electrophysiology. Dinitrophenol (200 μM), which uncouples oxidative phosphorylation, induced opening of K_{ATP} channels and Ca^{2+} loading. Glyburide (6 μM) which reduced the opening of K_{ATP} channels, aggravated Ca^{2+} loading only when applied to dinitrophenol-pretreated myocytes but not when applied with dinitrophenol treatment. We conclude that a blocker of K_{ATP} channels has differential effects upon dinitrophenol-induced intracellular Ca^{2+} loading, which appear to depend upon the stage of metabolic insult.

Keywords: ATP-sensitive K^+ channel; Ca^{2+}, intracellular; Chemical hypoxia; Dinitrophenol; Cardiac cell; Glyburide; Fluo-3

1. Introduction

A role for ATP-sensitive K^+ (K_{ATP}) channels in protecting the myocardium from ischemic insult has been proposed (Parratt and Kane, 1994; Yao and Gross, 1994; Grover et al., 1995). This notion is based upon the observation that activators of K_{ATP} channels can enhance or mimic intrinsic cardioprotective mechanisms, such as ischemic preconditioning (Parratt and Kane, 1994; Yao and Gross, 1994; Grover et al., 1995). Furthermore, clinical and epidemiological studies have suggested an association between sulfonylurea therapy, which presumably blocks K_{ATP} channels, and an increase in mortality due to myocardial infarction (The University Group Diabetes Program, 1970). However, the assumption that opening of K_{ATP} channels during metabolic impairment is cardioprotective could not be confirmed as results regarding the action of sulfonylureas on the myocardium have been inconsistent (Thornton et al., 1993; Yao and Gross, 1994).

Such inconsistency in the ability of sulfonylureas to block the protective effects of preconditioning may be related to various factors, including a reduction in the efficacy with which sulfonylureas inhibit K_{ATP} channels under conditions of extreme metabolic stress (Findlay, 1993). Moreover, the effect of pharmacological K_{ATP} channel blockade has been examined primarily in whole heart or multicellular preparations (Thornton et al., 1993; Yao and Gross, 1994), and therefore the effect of K_{ATP} channel modulation on neuronal, vascular, or interstitial tissues, as well as systemic effects, cannot be excluded (Armstrong et al., 1995). Hence, the contribution of cardiac K_{ATP} channels to mechanisms of cardioprotection at the cellular level remains controversial.

The aim, therefore, of the present study was to assess the effect of a K_{ATP} channel antagonist on cytoprotective mechanisms against metabolic impairment in single cardiomyocytes. Towards this goal, we examined the effect of glyburide, a prototype sulfonylurea and cardiac K_{ATP} channel blocker (Findlay, 1992), on Ca^{2+} loading induced by metabolic impairment in isolated, ventricular, myocytes. Metabolic inhibition was induced by 2-4-dinitrophenol, which is known to uncouple oxidative phosphorylation (Allard et al., 1995), and cytosolic Ca^{2+} concentra-

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tion was measured as an indicator of cell injury (Tani, 1990). We report that glyburide can have a dual effect upon chemical hypoxia-induced Ca^{2+} loading, the outcome of which appears to depend upon the stage of cellular metabolic compromise.

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. The aorta was cannulated, and following cardiotomy, the heart retrogradely perfused (at 37°C) with the following solutions (in mM): (1) Tyrode: NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, HEPES-NaOH 5.5 (pH 7.4) for 5–10 min; (2) nominally Ca^{2+}-free Tyrode (2–5 min); (3) nominally Ca^{2+}-free Tyrode supplemented with collagenase (0.04 g per 100 ml, Sigma type I, Sigma Chemical Co., St. Louis, MO, USA) (30 min); (4) high K^+/low Cl^- solution: 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) for 5 min. The heart was, then, stored in the high K^+/low Cl^- solution at 4°C for 120 min. Single cells were isolated by agitating a small piece of dissected ventricle in a culture dish filled with Tyrode. Experiments were performed with the approval of the Institutional Animal Care and Use Committee, Mayo Foundation.

2.2. Epifluorescent digital imaging

Cardiomyocytes were loaded, at room temperature, with 3 μM of the acetoxymethyl ester form of Fluo-3 (Fluo-3AM; Molecular Probes, Eugene, OR, USA), a Ca^{2+}-selective fluorescent probe dissolved in dimethylsulfoxide and pluronic acid (López et al., 1996). In a separate series of experiments, cardiomyocytes were loaded with 5 μM of the ratiometric dye Fura-2 acetoxymethylester (Fura-2AM, Molecular Probes) to quantify the resting intracellular Ca^{2+} concentration. Fluo-3AM- or Fura-2AM-loaded cardiomyocytes were transferred to a coverslip mounted on the perforated bottom of an experimental chamber (1.5 ml) placed on the stage of an epifluorescent microscope, and superfused with Tyrode. Single, rod-shaped ventricular myocytes with clear striations were imaged, at 37°C, by digital epifluorescent microscopy using an inverted microscope (Zeiss Axiovert-135 TV) with a 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. In cells loaded with Fura-2AM, an estimate of the Ca^{2+} concentration ($[Ca^{2+}]$) was obtained using the equation:

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

where R is the actually recorded fluorescence ratio (340/380 nm); R_{min} and R_{max} fluorescence ratios when extracellular Ca^{2+} was removed (using 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 F_{min}/F_{max} at 380 nm. To obtain F_{min} and F_{max}, Fura-2AM-loaded cardiac cells were exposed to the calcium ionophore, 4-bromo A-23187. To prevent cell contraction in permeabilized cells exposed to high concentrations of extracellular Ca^{2+}, myocytes were pretreated with carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (2 μM) and 2,3-butaneodione monoxime (40 mM). An estimate of the increase in $[Ca^{2+}]_i$ as a function of Fluo-3 fluorescence was calculated from the equation:

$$[Ca^{2+}] = \frac{F - F_{min}}{F_{max} - F} \cdot K_d + [Ca^{2+}]_r$$

where F is the observed fluorescence, F_{min} and F_{max} represent the fluorescence in the absence of Ca^{2+} (extracellular Ca^{2+} was removed and 3 mM EGTA added to the extracellular solution) and at high $[Ca^{2+}]$ (3 mM $CaCl_2$), respectively. To obtain F_{min} and F_{max}, cells were exposed to the ionophore, A-23187 (see above). $[Ca^{2+}]_r$ is the resting $[Ca^{2+}]_i$ concentration determined in Fura-2-loaded ventricular myocytes (78 ± 11 nM; $n = 36$), and K_d the Ca^{2+} dissociation constant of Fluo-3 (422 nM).

2.3. Single channel recording

The gigaohm-seal patch-clamp technique was employed in the cell-attached configuration, and single channel currents recorded at 37°C. Pipettes were filled with (in mM): KCl 140, $CaCl_2$ 1, $MgCl_2$ 1, HEPES-KOH 5 (pH 7.4), and their resistance ranged between 5 and 7 MΩ. The tips of electrodes were coated with Sylgard and fire-polished. Cardiomyocytes were superfused (in mM) with: KCl 140, $MgCl_2$ 1, EGTA-KOH 5, HEPES-KOH 5 (pH 7.4). Channel activity was measured using a patch-clamp amplifier (Axopatch-1C, Axon Instruments, Foster City, CA, USA) and monitored on-line on a high-gain digital storage oscilloscope (VC-6025; Hitachi, Tokyo, Japan). The holding potential was −60 mV (i.e. +60 mV command potential to the cell-attached electrode). Data were stored on tape using a PCM converter system (VR-10, Instrutech; New York, NY, USA) reproduced, low-pass filtered at 1.5 kHz

(−3 dB) by a Bessel filter (Frequency Devices 902; Haverhill, MA, USA), sampled at 4 kHz, and analyzed off-line with a computer (Everex Step) and the analysis program 'BioQuest' (designed by A.E.A). The degree of channel activity was assessed, under respective experimental conditions, by digitizing segments of the current records. Channel activity was quantified in terms of NP_o, where N represents the number of channels in the patch, and P_o the probability of each channel to be open.

2.4. Drugs

2,4-Dinitrophenol and glyburide were purchased from Sigma, and dissolved in dimethylsulfoxide. The final concentration of dimethylsulfoxide never exceeded 0.1%.

2.5. Statistics

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

3. Results

3.1. Dinitrophenol induces cytosolic Ca^{2+} loading in single cardiomyocytes

Under resting conditions, intracellular Ca^{2+} concentration is low in ventricular cardiomyocytes (Fig. 1A, upper frame). Exposure to dinitrophenol, an uncoupler of oxidative phosphorylation, induced a significant increase in intracellular Ca^{2+} concentration (Fig. 1A, lower frame). On average, the intracellular Ca^{2+} concentration at rest was estimated at 127.6 ± 8.4 nM, but reached 285.7 ± 8.0 nM following exposure to 200 μM of dinitrophenol (n = 12; $P < 0.01$, Fig. 1A1). Half-maximal and peak increase in cytosolic Ca^{2+} concentration were reached within 10.3 ± 1.1 and 15.3 ± 1.9 min, respectively, following addition of 200 μM dinitrophenol ($n = 12$; Fig. 1A). Thus, metabolic impairment induced by dinitrophenol is associated with moderate and gradual cytosolic Ca^{2+} loading.

3.2. Dinitrophenol induces opening of K_{ATP} channels in single cardiomyocytes

Prior to addition of dinitrophenol, no K_{ATP} channel activity could be recorded in the cell-attached patch configuration (Fig. 1B). Exposure of cardiomyocytes to 200 μM dinitrophenol gradually induced K_{ATP} channels to open (Fig. 1B). Channel opening occurred in bursts (Fig. 1B), and current-voltage relationships for dinitrophenol-induced channel openings revealed a single channel conductance which ranged between 70 and 90 pS (not illustrated), characteristic of myocardial K_{ATP} channels (Trube and Hescheler, 1984; Findlay, 1993). On average, NP_o (see Materials and methods) was essentially zero in the absence, but reached an apparent maximal value of 3.2 ± 0.4 in the presence of 200 μM dinitrophenol ($n = 7$; Fig. 1B). The peak level of K_{ATP} channel activity was reached within 2–8 min following addition of dinitrophenol ($n = 7$; Fig. 1B), and the time required for half-maximal channel activation was 4 ± 1 min ($n = 7$). Thus, dinitrophenol induces a gradual opening of myocardial K_{ATP} channels.

3.3. Glyburide reduces K_{ATP} channel openings in dinitrophenol-treated single cardiomyocytes

In cardiomyocytes co-exposed to dinitrophenol (200 μM) and glyburide (6 μM) no significant opening of K_{ATP} channels could be recorded, at least within 10-min-long exposures to both drugs (Fig. 1C). Accordingly, K_{ATP} channel activity, expressed as NP_o, remained essentially zero during co-additions of dinitrophenol and glyburide within this time frame. In myocytes pre-exposed to 200 μM dinitrophenol, in which the uncoupler of oxidative phosphorylation induced maximal K_{ATP} channel activity, further addition of glyburide (6 μM) reduced K_{ATP} channel openings (Fig. 1D). In these dinitrophenol-pretreated myocytes, the NP_o was 3.1 ± 0.4 in dinitrophenol alone, and decreased to 0.08 ± 0.02 after further addition of glyburide ($n = 7$).

3.4. Dual effect of glyburide on dinitrophenol-induced Ca^{2+} loading in single cardiomyocytes

Dinitrophenol (200 μM), added concomitantly with glyburide (6 μM), induced intracellular Ca^{2+} loading (Fig. 2A). In the presence of both dinitrophenol and glyburide, the estimated intracellular Ca^{2+} concentration increased to 304.0 ± 9.4 nM ($n = 5$) (Fig. 2A1). This value was not significantly different from that obtained (Fig. 1A) when cardiomyocytes were exposed to dinitrophenol alone (285.7 ± 8 nM, $n = 12$, $P > 0.05$). Thus, glyburide had no apparent effect on dinitrophenol-induced Ca^{2+} loading in cardiomyocytes concomitantly exposed to both drugs.

However, in cardiomyocytes in which 10-min pre-exposure to dinitrophenol (200 μM) already induced modest Ca^{2+} loading (Fig. 2B, frames 1 and 2), further addition of glyburide (6 μM) was associated with a dramatic increase in intracellular Ca^{2+} concentration (Fig. 2B, frame 3). This increase in Ca^{2+} concentration led to cell contracture (Fig. 2B, frames 4 and 5). The estimated maximal value of cytosolic Ca^{2+} concentration following addition of glyburide in dinitrophenol-pretreated myocytes was 2466 ± 211 nM ($n = 6$; Fig. 2B1). This value was almost 10 times higher than that induced by dinitrophenol alone (285.7 ± 8 nM, $n = 12$, $P < 0.01$), or that induced by dinitrophenol plus glyburide when the sulfonylurea was added concomitantly with the uncoupler of oxidative phosphorylation

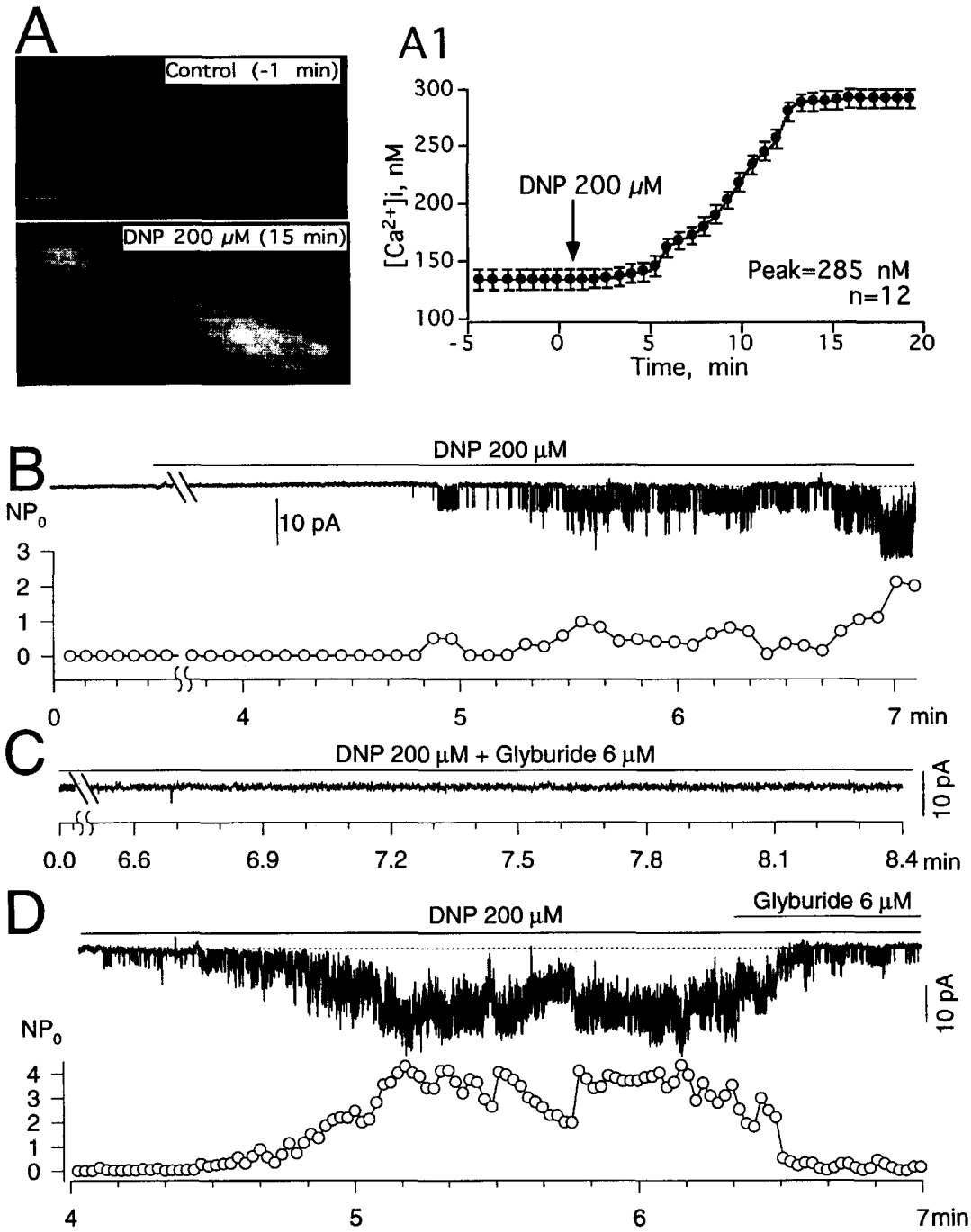

Fig. 1. A–B: The uncoupler of oxidative phosphorylation, dinitrophenol (DNP), induces moderate intracellular Ca^{2+} loading and opening of ATP-sensitive K^+ (K_{ATP}) channels in single ventricular cardiomyocytes. A: Epifluorescent digital images obtained from a, Fluo-3-loaded, cardiomyocyte, 1 min prior (upper frame), and 15 min following addition of 200 μM dinitrophenol to the solution bathing the myocyte (lower frame). Horizontal bar indicates 20 μm. A1: Average changes in the estimated intracellular Ca^{2+} concentration expressed as a function of time. Average peak value induced by exposure to 200 μM dinitrophenol was 285.7 ± 8.0 nM (n = 12). B: Upper trace: continuous channel record obtained in the cell-attached configuration of the patch-clamp technique. Lower trace: NP_0 values corresponding to upper trace record calculated over 4.8-s-long intervals. C–D: Glyburide, a sulfonylurea, reduces the opening of ATP-sensitive K^+ (K_{ATP}) channels in dinitrophenol-treated single ventricular myocytes. C: Continuous channel record obtained in the cell-attached configuration of the patch-clamp technique from a myocyte concomitantly exposed to dinitrophenol (200 μM) and glyburide (6 μM). D: Upper trace: continuous channel record obtained in the cell-attached configuration from a myocyte first exposed to dinitrophenol (200 μM) alone, and then concomitantly to dinitrophenol and glyburide (6 μM). Lower trace: corresponding NP_0 values calculated over 4.8-s-long intervals. In B–D, dotted lines correspond to zero current level, and holding potential was −60 mV. Downward current deflections indicate inward current. In all figures, time 0 corresponds to the time of addition of dinitrophenol.

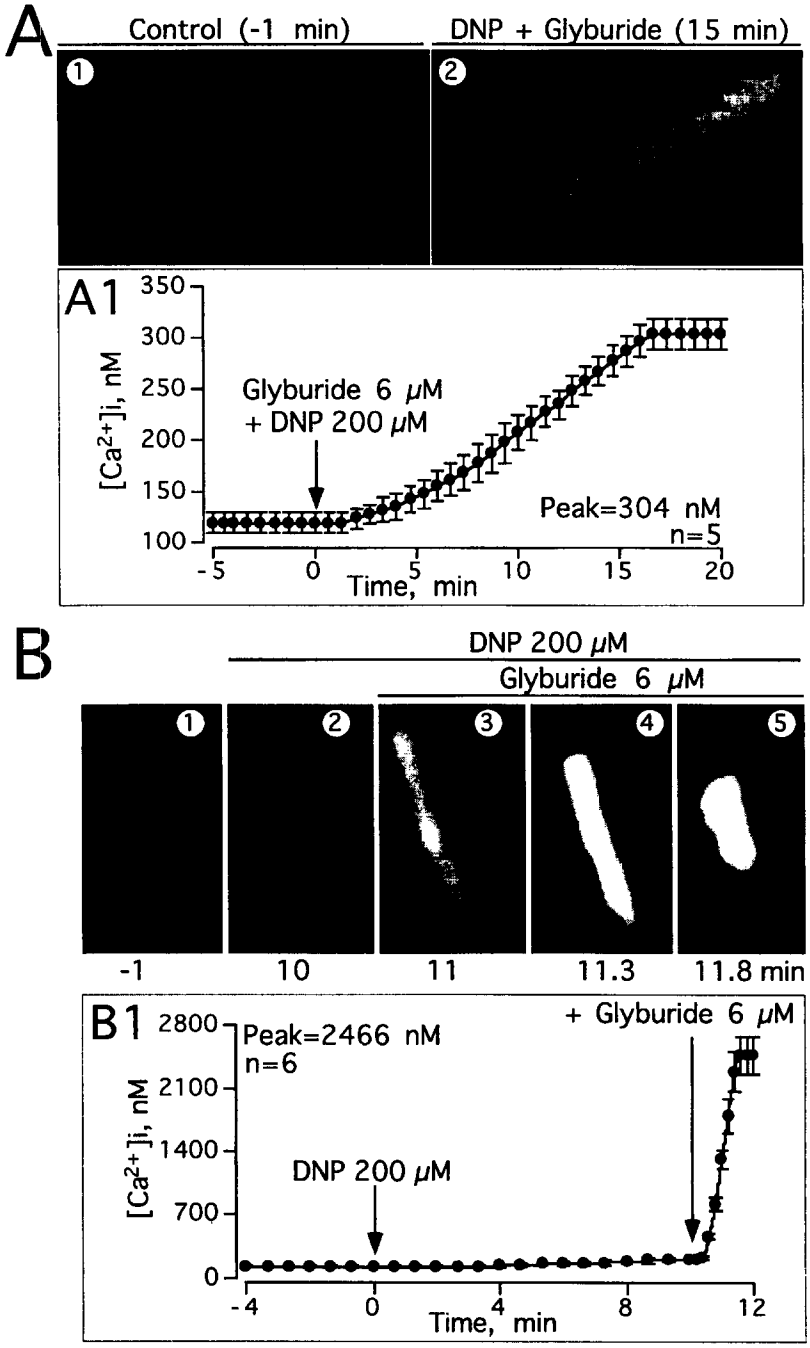

Fig. 2. Differential effect of glyburide on dinitrophenol (DNP)-induced Ca^{2+} loading depends on the stage of dinitrophenol action. A: Glyburide, added concomitantly with dinitrophenol, does not apparently aggravate dinitrophenol-induced Ca^{2+} loading in ventricular myocytes. Epifluorescent digital images obtained from a, Fluo-3-loaded, cardiomyocyte 1 min prior (frame 1), and 15 min following addition of 200 μM dinitrophenol plus 6 μM glyburide (frame 2). Horizontal bar indicates 20 μm. A1: Average changes in the estimated intracellular Ca^{2+} concentration expressed as a function of time. Average peak value induced by exposure to 200 μM dinitrophenol plus 6 μM glyburide was 304 ± 9.4 nM ($n = 5$). B: Glyburide, added to dinitrophenol-pretreated myocytes, aggravates dinitrophenol-induced Ca^{2+} loading. Epifluorescent digital images obtained from a, Fluo-3-loaded, cardiomyocyte 1 min prior (frame 1), and 10 (frame 2), 11 (frame 3), 11.3 (frame 4) and 11.8 min (frame 5) following addition of dinitrophenol (200 μM). The apparent Ca^{2+} concentration was 121, 219, 1596, 2325 and 2562 nM in frames 1–5, respectively. Note that glyburide (6 μM) was added prior to frame 3. Also, note the rod to square transformation of the myocyte indicative of chemical hypoxia-induced contracture. Horizontal bar indicates 20 μm. B1: Average changes in the estimated intracellular Ca^{2+} concentration expressed as a function of time. Average peak value induced by exposure, of a dinitrophenol-pretreated cardiomyocyte, to 200 μM dinitrophenol plus 6 μM glyburide was 2466 ± 211 nM ($n = 6$). In all figures, time 0 corresponds to the time of addition of dinitrophenol.

(304 ± 9.4 nM, $n = 5$, $P < 0.01$). Thus, glyburide could aggravate dinitrophenol-induced Ca^{2+} loading when applied to already metabolically compromised single cardiomyocytes.

4. Discussion

Cardiac myocytes are highly dependent upon mitochondrial oxidative phosphorylation. Therefore, impairment of this mechanism of ATP production, which occurs not only with dinitrophenol treatment but also during ischemia, can lead to metabolic cellular dysfunction (Ferrari et al., 1993). The finding that dinitrophenol, an inhibitor of oxidative phosphorylation, induces opening of K_{ATP} channels, and intracellular Ca^{2+} loading in single cardiomyocytes is in accord with previous reports in which other metabolic poisons, as well as hypoxic or ischemic injury, have been shown to elevate the intracellular concentration of Ca^{2+} (Steenbergen et al., 1993) and open myocardial K_{ATP} channels (Weiss and Venkatesh, 1993). The observed Ca^{2+} loading could have been the consequence of both perturbations of sarcolemmal Ca^{2+} transport systems leading to an increase in Ca^{2+} influx, as well as release of Ca^{2+} from intracellular Ca^{2+} stores, including mitochondrial Ca^{2+} pools as proposed for other models of myocardial injury (Tani, 1990; Steenbergen et al., 1993; Chacon et al., 1994). In the present study, the concentration of cytosolic Ca^{2+} estimated in response to dinitrophenol (200 μM) was rather modest (< 1 μM), when considering that it is at concentrations of intracellular Ca^{2+} of 1 μM or greater that Ca^{2+} overload leads to disruption in energy transduction, and cell death occurs (Tani, 1990). It is conceivable, therefore, that endogenous protective mechanism(s), such as the opening of K_{ATP} channels, may limit the effect of dinitrophenol on the severity of Ca^{2+} loading (< 1 μM).

Although extensively studied, the role of K_{ATP} channels during metabolic insult is not fully understood (Weiss and Venkatesh, 1993; Yao and Gross, 1994). While it has been reported that the opening of sarcolemmal K_{ATP} channels is a cause of marked cellular loss of K^+ during ischemia and hypoxia and could be proarrhythmic (Wilde et al., 1990; Weiss and Venkatesh, 1993), activation of K_{ATP} channels does appear to be protective against myocardial infarction in whole heart preparations (Yao and Gross, 1994). The mechanism by which K_{ATP} channel opening protects the myocardium from injury has not been determined, but it has been postulated that an efflux of K^+ will hyperpolarize the membrane potential resulting in a reduction in net Ca^{2+} influx, and a decrease in the metabolic demands of a cardiomyocyte (Parratt and Kane, 1994). However, additional mechanisms functional at levels remote from the sarcolemma have been proposed suggesting that sarcolemmal movement of K^+ may not be necessary for cardioprotection, and that K_{ATP} channels located in intracellular sites, such as the mitochondrial inner membrane, may play a role in cardioprotection (Grover et al., 1995).

Whether opening of K_{ATP} channels, as a consequence of metabolic inhibition, is actually cytoprotective against Ca^{2+} loading within quiescent single cardiomyocytes was examined, herein, by testing the action of glyburide. First, we established that under our experimental conditions of modest stress, K_{ATP} channels remain sensitive to the blocking action of glyburide. This is in accord with previous findings that openings of K_{ATP} channels during the early stages of metabolic insult were glyburide-sensitive, in contrast to glyburide-insensitive current which developed under conditions of extreme metabolic insult (Findlay, 1993). Concomitant exposure of cardiomyocytes to glyburide and dinitrophenol apparently did not alter the effect of dinitrophenol on intracellular Ca^{2+} loading. This lack of glyburide action is in accord with the concept that inhibition of K_{ATP} channels does not affect the rise of intracellular Ca^{2+} concentration during myocardial ischemia (Steenbergen et al., 1993) suggesting that glyburide-insensitive mechanisms of cardioprotection predominate under this experimental condition. In contrast, after dinitrophenol had already induced partial metabolic inhibition and modest increase in intracellular Ca^{2+}, glyburide further increased Ca^{2+} loading, which then exceeded 2 μM, a concentration incompatible with normal cell function leading to irreversible cell contracture (Tani, 1990). This could be interpreted to suggest that following the onset of partial metabolic inhibition, a glyburide-sensitive mechanism may assume a central role in protection against Ca^{2+} loading. Thus, it appears that glyburide, a known antagonist of K_{ATP} channels, could have a dual effect upon intracellular Ca^{2+} loading induced by metabolic stress. Such a finding is in accord with the hypothesis that the ability of sulphonylurea drugs to block endogenous myocardial protection depends on the stage of the ischemic insult (Armstrong et al., 1995).

Further investigation is required to define the precise mechanism(s) responsible for the dual effect of glyburide on metabolic inhibition-induced Ca^{2+} loading. Regardless of the underlying mechanism, our findings may provide an explanation for the previously reported inconsistency in the action of sulfonylureas on the myocardium under different pathophysiological conditions, and could imply a dynamic pharmacological regulation of cardiac cell function under conditions of metabolic compromise.

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