

Preferential regulation of rabbit cardiac L-type Ca^{2+} current by glycolytic derived ATP via a direct allosteric pathway

Vito A. Losito, Robert G. Tsushima, Roberto J. Diaz*, Gregory J. Wilson* and Peter H. Backx

*Department of Physiology, University of Toronto and Center for Cardiovascular Research, Toronto General Hospital and *Divisions of Cardiovascular Research and Pathology, The Hospital for Sick Children, Toronto, Ontario, Canada*

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1. The activity of Ca^{2+} channels is regulated by a number of mechanisms including direct allosteric modulation by intracellular ATP. Since ATP derived from glycolysis is preferentially used for membrane function, we hypothesized that glycolytic ATP also preferentially regulates cardiac L-type Ca^{2+} channels.
2. To test this hypothesis, peak L-type Ca^{2+} currents (I_{Ca}) were measured in voltage-clamped rabbit cardiomyocytes during glycolytic inhibition (2-deoxyglucose + pyruvate), oxidative inhibition (cyanide + glucose) or both (full metabolic inhibition; FMI).
3. A 10 min period of FMI resulted in a 40.0% decrease in peak I_{Ca} at +10 mV (-5.1 ± 0.6 versus -3.1 ± 0.4 pA pF $^{-1}$; $n = 5$, $P < 0.01$). Similar decreases in peak I_{Ca} were observed during glycolytic inhibition using 2-deoxyglucose (-6.2 ± 0.2 versus -3.7 ± 0.2 pA pF $^{-1}$; $n = 5$, $P < 0.01$) or iodoacetamide (-6.7 ± 0.3 versus -3.7 ± 0.2 pA pF $^{-1}$; $n = 7$, $P < 0.01$), but not following oxidative inhibition (-6.2 ± 0.4 versus -6.4 ± 0.3 pA pF $^{-1}$; $n = 5$, n.s.). The reduction in I_{Ca} following glycolytic inhibition was not mediated by phosphate sequestration by 2-deoxyglucose or changes in intracellular pH.
4. Reductions in I_{Ca} were still observed when inorganic phosphate and creatine were included in the pipette, confirming a critical role for glycolysis in I_{Ca} regulation.
5. With 5 mM MgATP in the pipette during FMI, peak I_{Ca} decreased by only 18.4% (-6.8 ± 0.6 versus -5.5 ± 0.3 pA pF $^{-1}$; $n = 4$, $P < 0.05$), while inclusion of 5 mM MgAMP-PCP (β,γ -methyleneadenosine 5'-triphosphate, Mg $^{2+}$ salt) completely prevented the decrease in peak I_{Ca} (-6.9 ± 0.3 versus -6.5 ± 0.3 pA pF $^{-1}$; $n = 5$, n.s.).
6. Together, these results suggest that I_{Ca} is regulated by intracellular ATP derived from glycolysis and does not require hydrolysis of ATP. This regulation is expected to be energy conserving during periods of metabolic stress and myocardial ischaemia.

In the myocardium, as well as other tissues, ATP synthesis and utilization are subcellularly localized (Lynch & Paul, 1987). Glycolytic enzymes are known to be associated with the cardiac sarcolemma (Pierce & Philipson, 1985; Hazen *et al.* 1994) and sarcoplasmic reticulum (Pierce & Philipson, 1985). Accordingly, ATP derived from glycolysis has been shown to preferentially support membrane function while ATP generated from oxidative phosphorylation supports contractile function (Weiss & Hiltbrand, 1985). For instance, glycolytic ATP has been shown to preferentially regulate cardiac, as well as skeletal, sarcoplasmic reticular Ca^{2+} transport (Xu *et al.* 1995). Furthermore, glycolytic ATP regulates the activity of both the sarcolemmal Ca^{2+} pump (Paul *et al.* 1989; Hardin *et al.* 1992) and the Na^+ - K^+ pump

(Lynch & Balaban, 1987; Campbell & Paul, 1992) in smooth muscle cells.

Preferential regulation of ATP-sensitive K^+ channels by glycolysis has been demonstrated in guinea-pig cardiac myocytes (Weiss & Lamp, 1987, 1989). Regulation of the ATP-sensitive K^+ channel by ATP is the prototype for a putative metabolic feedback of cellular excitability and contractility. As intracellular ATP concentrations decrease, ATP-sensitive K^+ channels open in a dose-dependent fashion in guinea-pig myocytes (Deutsch & Weiss, 1993) resulting in K^+ efflux, shortening of the action potential and reduced excitability (Deutsch *et al.* 1991; Weiss *et al.* 1992). Ultimately, reduction of excitability reduces contractility

preserving intracellular ATP and conferring protection against metabolic stress (Critz *et al.* 1997).

Like ATP-sensitive K⁺ channels, L-type Ca²⁺ channels are also regulated by ATP. Intracellular injection of ATP in whole-cell patch-clamp studies of guinea-pig myocytes resulted in a 40–60% prolongation of action potential duration (Taniguchi *et al.* 1983), which correlated with a corresponding increase in L-type Ca²⁺ current (I_{Ca}) (Irisawa & Kokubun, 1983). One potential mechanism for this regulation is that intracellular ATP acts as a substrate for phosphorylation of the Ca²⁺ channel by protein kinases (for a review see Hilgemann, 1997). Alternatively, intracellular ATP also regulates Ca²⁺ channel activity directly via an allosteric mechanism not requiring ATP hydrolysis (O'Rourke *et al.* 1992; Yazawa *et al.* 1997; Yokoshiki *et al.* 1997) as originally proposed by Reuter & Scholtz (1977). In contrast to the regulation of ATP-sensitive K⁺ channels by free ATP, direct regulation of I_{Ca} requires MgATP (Backx *et al.* 1991; O'Rourke *et al.* 1992). Direct phosphorylation-independent regulation of I_{Ca} has also been demonstrated in smooth muscle cells from rat mesenteric artery (Yokoshiki *et al.* 1997) and this regulation appeared to rely preferentially on ATP derived from glycolysis (Lorenz & Paul, 1997).

Given that cardiac L-type Ca²⁺ channels are regulated by ATP and that glycolytic ATP has been shown to preferentially influence membrane proteins, we hypothesized that ATP derived from glycolysis would have a more direct influence on cardiac L-type Ca²⁺ channels compared with oxidative phosphorylation. In addition, we hypothesized that this regulation may involve a direct phosphorylation-independent mechanism. We tested these hypotheses in whole-cell voltage-clamped rabbit ventricular myocytes, which were exposed to glycolytic inhibition, oxidative inhibition or both glycolytic and oxidative inhibition (full metabolic inhibition; FMI). Our results establish that peak I_{Ca} is preferentially regulated by glycolytic ATP via a direct allosteric pathway.

METHODS

Preparation of single cells

Single ventricular cells were obtained from rabbit hearts (New Zealand White, 2–3 kg) using a modification of a previously described technique (Backx & Marban, 1993). All experiments were carried out with the approval of the Animal Care Committee at the Toronto General Hospital in accordance with the guidelines set by the Canadian Council for Animal Care. Briefly, each rabbit was anaesthetized with a mixed solution of pentobarbitone (60 mg kg⁻¹) and heparin (200 i.u. kg⁻¹) given as two intravenous bolus doses separated by 5 min. Before respiratory arrest occurred, the chest was opened through a mid-line sternotomy and the heart was quickly excised, mounted by the aortic root onto a cannula and retrogradely perfused with a modified Hepes-Tyrode solution with 1.0 mM Ca²⁺ and bubbled with 100% O₂ at 37 °C for 2–3 min to wash out all blood residue. The heart was then perfused with a nominally Ca²⁺-free Hepes-Tyrode solution for 10 min and finally with the same Ca²⁺-free solution containing 200 U ml⁻¹ collagenase Type II (Worthington Biochemical Corporation, Lakewood, NJ,

USA) for 25–30 min. The enzyme solution was subsequently washed out with a high-K⁺ solution for 5 min. The ventricles were then removed and dissected into small pieces and single cells were isolated by gentle mechanical agitation. Cells were filtered through a nylon mesh, resuspended in high-K⁺ solution with 2% bovine serum albumin (Sigma-Aldrich) and used within 8 h. Calcium-tolerant, rod-shaped cells with clear, regular cross-striations and no visible blebs were used for electrophysiological recordings.

Solutions and drugs

The modified Hepes-Tyrode solution for cell isolation contained (mM): NaCl, 140; MgCl₂, 1; Hepes, 10; KCl, 4; CaCl₂, 1; D-glucose, 5; pH adjusted to 7.4 with NaOH. The high-K⁺ cell storage solution contained (mM): potassium glutamate, 120; KCl, 20; Hepes, 20; MgCl₂, 1; D-glucose, 10; and K-EGTA, 0.3; pH 7.4. The control bath (external recording) solution contained (mM): NaCl, 145; MgCl₂, 1; CsCl, 5; Hepes, 10; CaCl₂, 1.8; pH adjusted to 7.4 with NaOH. D-Glucose (10 mM) and pyruvic acid (2 mM) were added to the control bath solution as substrate for glycolysis and oxidative phosphorylation, respectively. The control pipette (internal recording) ATP-free solution contained (mM): CsCl, 145; Hepes, 10; MgCl₂, 1; EGTA, 5; pH adjusted to 7.2 with CsOH; the calculated free Mg²⁺ concentration under these conditions was 0.79 mM (CHELATOR Version 1.00; Schoenmakers *et al.* 1992).

FMI was achieved by the addition of 10 mM 2-deoxyglucose to inhibit glycolysis and 2 mM sodium cyanide to inhibit oxidative phosphorylation, in the absence of substrate (i.e. glucose and pyruvate). Inhibition of glycolysis alone was achieved by inclusion in the bath solution of 10 mM 2-deoxyglucose and 2 mM pyruvic acid (substrate for oxidative phosphorylation). In addition to competitively inhibiting glucose utilization, 2-deoxyglucose also inhibits glycogen phosphorylase (Dringen & Hamprecht, 1993; Oikonomakos *et al.* 1995) and activates glycogen synthase (Carabaza *et al.* 1992). To determine the specificity of 2-deoxyglucose we used 100 μM iodoacetamide (IAA), a known inhibitor of glyceraldehyde-3-phosphate dehydrogenase, to inhibit glycolysis. Inhibition of oxidative phosphorylation alone was achieved by inclusion of 2 mM sodium cyanide in the control bath solution containing 10 mM glucose as substrate for glycolysis. In a separate set of experiments 5 mM ATP or 5 mM β,γ-methyleneadenosine 5'-triphosphate (AMP-PCP) complexed as Mg²⁺ salts was included in the control pipette solution. The estimated free Mg²⁺ concentration in the pipette solutions was 1.1 mM for MgATP or MgAMP-PCP (CHELATOR Version 1.00). To investigate the role of the creatine kinase, experiments were conducted with either 1.2 mM NaH₂PO₄ in the bath and 3.0 mM NaH₂PO₄ in the pipette solution, or the same concentrations of NaH₂PO₄ and 20 mM creatine in the pipette solution.

Glibenclamide (1 μM) was added to the bath solution (from a 10 mM stock dissolved in 100% DMSO) to inhibit ATP-sensitive K⁺ channels during metabolic inhibition. R(+)-methylindazole (IAA94; 10 μM) or 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB; 1 μM) were added to the bath solution to block Cl⁻ channels (from a 50 mM stock dissolved in 100% ethanol). IAA94 and NPPB were purchased from Research Biochemicals International. Glibenclamide, creatine, ATP, AMP-PCP, iodoacetamide, cyanide, 2-deoxyglucose and all other analytical grade chemicals were purchased from Sigma-Aldrich.

Electrophysiological recording

Cells were transferred into a 1 ml perfusion bath placed on the stage of an inverted microscope and superfused with control bath solution containing glucose and pyruvate at a rate of 1–2 ml min⁻¹. All experiments were performed at room temperature (20–22 °C).

The gigaseal, whole-cell voltage-clamp configuration of the patch-clamp technique (Hamill *et al.* 1981) using an Axopatch 200A or 200B amplifier (Axon Instruments) was utilized. A ground silver–silver chloride wire enclosed in an agar bridge (Backx & Marban, 1993) was used to avoid oxidation of the silver upon wash-in of cyanide and subsequent junction potential shifts. Recording pipettes were prepared from thin-walled borosilicate glass (1.5 mm diameter; World Precision Instruments) using a Flaming–Brown micropipette puller (model P-87; Sutter Instruments). The pipette tip was heat polished with a heating filament (model MF-83; Narishige, Japan) and when filled with internal solution, tip resistance was typically 2–3 M Ω . Uncompensated series resistance was typically 2–6 M Ω and compensation was 60–70%. After membrane rupture, the cell capacitance was estimated automatically by custom-designed computer software and by integrating the area under the capacity transient generated from a 20 mV depolarization from a holding potential of –40 mV. Currents were digitized at 10 kHz, filtered at 2 kHz and stored off-line for analysis.

To isolate I_{Ca} , internal and external K^+ was replaced by Cs^+ , Cl^- currents were blocked with 10 μ M IAA94 or 1 μ M NPPB, potent inhibitors of Cl^- channels (Pope *et al.* 1991), and Na^+ channels were inactivated by a 100 ms depolarizing prepulse to –40 mV. After completion of metabolic inhibition, nifedipine (10 μ M) or Cd^{2+} (300 μ M) was added to the bath solution to obtain nifedipine- or Cd^{2+} -sensitive I_{Ca} . Peak I_{Ca} was sampled every 5 s by 200 ms depolarizing pulses to 0 mV from a holding potential of –80 mV, after 100 ms prepulses to –40 mV. Peak I_{Ca} I – V relationships were measured between –40 and +80 mV in 10 mV increments. During the time course of our metabolic inhibition experiments, leak currents were estimated by measuring the current recorded at the holding potential (–80 mV) and the prepulse potential (–40 mV).

Experimental protocols and data analysis

This study consisted of eight groups of cells each representing different metabolic interventions in the absence or presence of ATP or AMP-PCP in the pipette solution and each cell was used as its own control. After making a gigaseal, gaining access into the myocytes and compensating for cell capacitance and series resistance, myocytes were repeatedly depolarized to 0 mV from a holding potential of –80 mV with a 100 ms prepulse to –40 mV. This was done for 5 min while the cells were superfused with external control solution containing glucose and pyruvate in order to allow complete cellular dialysis and endogenous run-down of I_{Ca} . Following the 5 min equilibration period, a control peak I_{Ca} I – V relationship (depolarizations from –40 to +80 mV) was recorded. This was followed immediately by application of bath solution containing the desired metabolic substrates and inhibitors, while the myocyte was again depolarized every 5 s. After a 10 min exposure to the metabolic inhibitors, a test I – V relationship was recorded and compared with the control I – V relationship. At the end of the protocol a nifedipine (10 μ M) or Cd^{2+} (300 μ M) current was collected.

The first group of cells was exposed to 2-deoxyglucose and sodium cyanide (FMI) in the absence of the metabolic substrates glucose and pyruvate. In the second group of myocytes, addition of 2-deoxyglucose in the continued presence of pyruvate to maintain oxidative phosphorylation exclusively inhibited glycolysis. The third group of cells was exposed to sodium cyanide to inhibit oxidative phosphorylation in the continued presence of glucose to support glycolysis. In a fourth and fifth group of myocytes, inorganic phosphate alone or inorganic phosphate and creatine, respectively, were utilized under control conditions and during glycolytic inhibition with 2-deoxyglucose. In a sixth group of cells

IAA (100 μ M) was used to inhibit glycolysis as opposed to 2-deoxyglucose. The above experiments on cells from groups one to six were conducted in the absence of exogenous ATP. The seventh group of cells was exposed to FMI while exogenous ATP was provided via the pipette. The eighth group of cells was also exposed to FMI but with AMP-PCP complexed with Mg^{2+} in the pipette solution.

Curve fitting

Custom-designed and commercially available Microcal Origin (Microcal Software Inc., Northampton, MA, USA) software was used to analyse data. Limiting maximal slope conductances were calculated by fitting the I – V data below +40 mV with the Boltzmann distribution function. The time of half I_{Ca} inactivation was calculated from the ratio of I_{Ca} at +10 mV (peak) and I_{Ca} at the end of the depolarizing step and is referred to as time to half I_{Ca} inactivation ($t_{1/2}$). All data are presented as means \pm s.e.m. and Student's t test for paired data was used for statistical analysis. An experimental α -level of $P < 0.05$ was considered statistically significant.

RESULTS

Metabolic inhibition in the absence of exogenous ATP

Figure 1A and B summarizes the changes in I_{Ca} due to endogenous run-down. In these experiments, myocytes were initially superfused with an external Tyrode solution (see Methods) containing 10 mM glucose and 2 mM pyruvate as substrates for glycolysis and oxidative phosphorylation, respectively, with no ATP added to the pipette. After gaining access, myocytes were dialysed for 2–3 min while series resistance was compensated. Next, from a holding potential of –80 mV, I_{Ca} was then recorded following prepulses to –40 mV followed by depolarizations to 0 mV applied every 5 s for a period of 5 min. This period allowed complete cell dialysis, equilibration and endogenous run-down of I_{Ca} . Figure 1A indicates that, under our conditions, I_{Ca} decreased by 10–20% within the first 3–4 min of recording. After 3–4 min, peak I_{Ca} remained relatively constant for at least 20 min as shown by the raw traces in Fig. 1A (inset). In fact, no significant difference was observed between mean peak I_{Ca} recorded at 0 mV 7–8 min after the whole-cell configuration was established (open circle; 6.3 ± 0.4 pA pF $^{-1}$) and 20 min later (filled circle; 5.8 ± 0.5 pA pF $^{-1}$) as illustrated in Fig. 1B. In all experiments in this study, we allowed the myocytes to equilibrate for 7–8 min prior to recording a control I – V relationship. Thereafter the effects of metabolic interventions were measured during the subsequent period, which was 10 min in duration.

Figure 1C shows the peak I_{Ca} amplitude before and after (indicated by the arrow) application of an external solution containing 10 mM 2-deoxyglucose and 2 mM sodium cyanide (FMI), inhibitors of glycolysis and oxidative phosphorylation, respectively. Note that a 10 min period of FMI (filled circle) resulted in a nearly twofold decrease in I_{Ca} compared with control (open circle). FMI did not affect current inactivation (i.e. $t_{1/2} = 33.2 \pm 1.3$ ms for control *versus* 30.5 ± 2.6 ms for FMI; $n = 5$, n.s.) as illustrated by

the raw traces in Fig. 1C (inset). Figure 1D shows the mean $I-V$ relationships taken before and 10 min after FMI. On average, FMI reduced I_{Ca} compared with control from -5.1 ± 0.6 to -3.1 ± 0.4 pA pF $^{-1}$ ($P < 0.01$, $n = 5$) at +10 mV, while mean maximum slope conductance (see Methods) was decreased from 159 pS in control to 108 pS.

In all the experiments shown in Fig. 1 and subsequent experiments, I_{Ca} is reported as the nifedipine- or Cd $^{2+}$ -subtracted current, where nifedipine or Cd $^{2+}$ was added at the end of the experimental protocol. This procedure is a valid measure of I_{Ca} provided that metabolic inhibition does not activate time-dependent leak currents. We used currents measured at -80 and -40 mV as indicators of the magnitude of leak currents during the time course of our experiments. A summary of leak currents for control, FMI, glycolytic and oxidative inhibition at -80 and -40 mV is shown in Fig. 2A. After a 10 min period of metabolic intervention, current at -80 mV was -314.1 ± 44.5 pA for FMI, -278.7 ± 52.8 pA for glycolytic inhibition and -334.8 ± 35.3 pA for oxidative inhibition, compared with

-339.4 ± 22.6 pA for control at the same time point ($n = 5$, n.s.). Current at -40 mV was -117.2 ± 7.8 pA for FMI, -129.0 ± 35.1 pA for glycolytic inhibition and -146.2 ± 21.8 pA for oxidative phosphorylation compared with -137.6 ± 29.2 pA for control ($n = 5$, n.s.). Figure 2B illustrates currents recorded in the presence of 10 μ M nifedipine from control and after metabolic inhibition. Following a 10 min period with control bath solution the mean current at +10 mV in the presence of nifedipine was -39.1 ± 12.2 pA compared with -35.4 ± 9.8 pA for FMI, -34.2 ± 9.8 pA for glycolytic inhibition and -36.2 ± 2.4 pA for oxidative inhibition ($n = 5$, n.s.). Therefore, under our experimental conditions metabolic inhibition did not activate time-dependent leak currents.

Figure 3A illustrates the changes in peak I_{Ca} before and after (indicated by the arrow) application of the glycolytic inhibitor 2-deoxyglucose (10 mM) in the presence of pyruvate as substrate for oxidative phosphorylation, without ATP added to the pipette solution. The protocols used in these experiments were the same as those described in Fig. 1. For

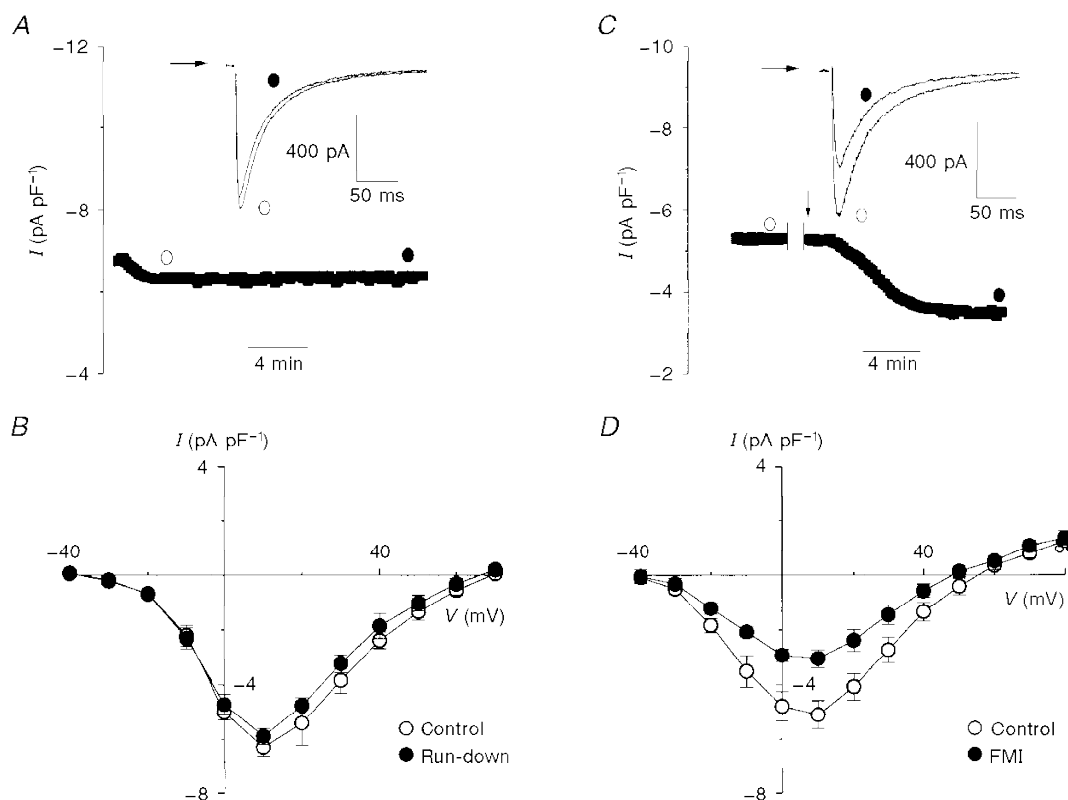


Figure 1. Effect of full metabolic inhibition on L-type Ca $^{2+}$ current

A, once the whole-cell configuration was achieved, I_{Ca} was sampled at 0.2 Hz and $I-V$ relationships were recorded at the times indicated by the open and filled circles. The typical raw traces (inset) indicate that I_{Ca} run-down was minor over the 10 min sampling period. B, mean $I-V$ relationships showing that sampling I_{Ca} at 0.2 Hz for 10 min did not significantly reduce I_{Ca} under our conditions ($n = 4$). C, I_{Ca} was sampled for 5 min before the indicated interruption in order to record a control $I-V$ relationship. This was followed by external wash-in (arrow) of 2-deoxyglucose (10 mM) and sodium cyanide (2 mM) (full metabolic inhibition, FMI). The raw traces (inset) demonstrate that I_{Ca} prior to FMI (open circle) was larger than I_{Ca} after FMI (filled circle). D, mean $I-V$ relationships which show that, on average, 10 min of FMI resulted in a 40% reduction of I_{Ca} at +10 mV ($n = 5$, $P < 0.01$).

the representative cell shown in Fig. 3A, a 10 min 2-deoxyglucose exposure period reduced I_{Ca} recorded at 0 mV by almost twofold. The raw traces in Fig. 3A (inset) demonstrate that exposure to 2-deoxyglucose (filled circle) caused a decrease in peak I_{Ca} compared with control (open circle). Similar to FMI, glycolytic inhibition reduced I_{Ca} without significantly affecting the time course of inactivation ($t_{1/2} = 30.4 \pm 1.2$ ms in control *versus* 30.4 ± 0.8 ms following glycolytic inhibition; $n = 5$, n.s.).

The mean $I-V$ relationships shown in Fig. 3B establish that 10 min of glycolytic inhibition resulted in a 43.3% reduction of I_{Ca} at +10 mV from -6.2 ± 0.2 to -3.7 ± 0.2 pA pF⁻¹ ($n = 11$, $P < 0.01$). In addition, Fig. 3B demonstrates that following a 30 min 2-deoxyglucose wash-out period I_{Ca} returned to control levels (-6.2 ± 0.2 pA pF⁻¹ for control *versus* -5.8 ± 0.3 pA pF⁻¹ after wash-out of 2-deoxyglucose; $n = 6$, n.s.). Similar to FMI, glycolytic inhibition resulted in a reduced mean slope conductance from 187 to 103 pS, which was completely reversed by wash-out of 2-deoxyglucose.

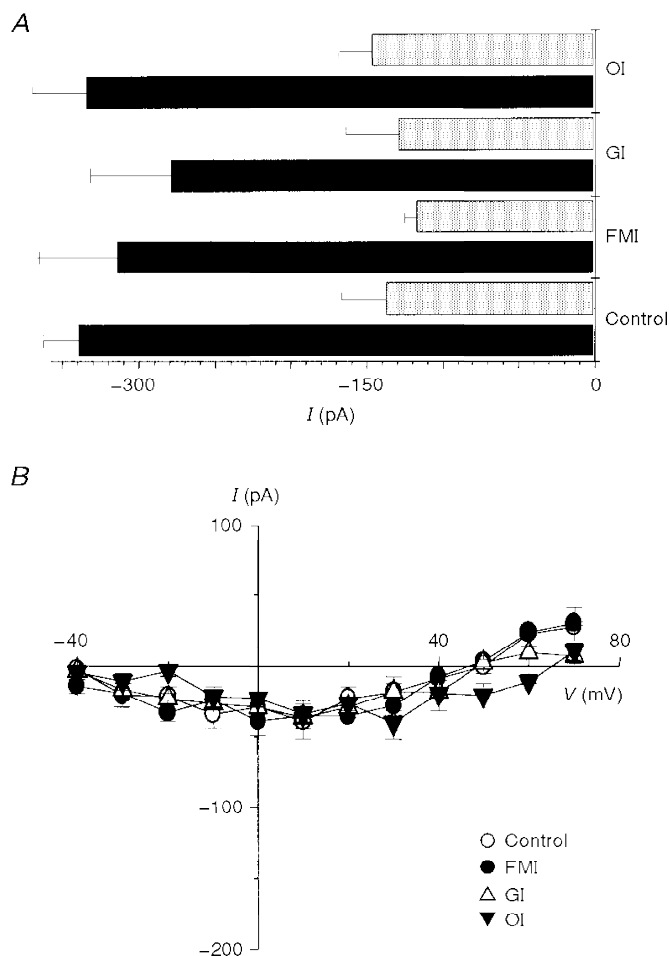
On the other hand, after 10 min of oxidative inhibition I_{Ca} increased modestly at 0 mV as shown in Fig. 3C. Examination of the raw traces in Fig. 3C (inset) showed that, as in the previous experiments, inactivation was not

changed by oxidative inhibition (i.e. $t_{1/2} = 32.2 \pm 1.3$ ms in control (open circle) *versus* 30.5 ± 1.6 ms following oxidative inhibition (closed circle); $n = 5$, n.s.). Figure 3D shows the mean $I-V$ relationships before and after application of cyanide. Clearly, the effect of cyanide was limited to a modest increase in peak I_{Ca} between -30 and 0 mV with no difference recorded at more positive voltages. At +10 mV, mean peak I_{Ca} was not altered by cyanide application (-6.2 ± 0.4 pA pF⁻¹ in control *versus* -6.4 ± 0.3 pA pF⁻¹ following oxidative inhibition; $n = 5$, n.s.) while the mean maximum slope conductances were virtually unchanged (i.e. 168 pS in control *versus* 161 pS after cyanide).

Like glucose, 2-deoxyglucose is phosphorylated to 2-deoxyglucose-6-phosphate (Oikonomakos *et al.* 1995). Due to the inability to metabolize 2-deoxyglucose-6-phosphate, the addition of 2-deoxyglucose could in principle sequester the pool of inorganic phosphate. This phosphate sequestration by 2-deoxyglucose could indirectly inhibit ATP production by the mitochondria and contribute to the reduction of I_{Ca} . Therefore, we conducted experiments in which we added 1.2 mM inorganic phosphate (NaH_2PO_4) to the bath solution and 3 mM NaH_2PO_4 to the pipette solution. The mean $I-V$ relationships shown in Fig. 4A demonstrate that, following a 10 min exposure to 2-deoxyglucose in the presence of NaH_2PO_4 , I_{Ca} decreased

Figure 2. Holding current in control and after various metabolic interventions

A, summary of holding current at -80 mV (■) and -40 mV (▨) for control, full metabolic inhibition (FMI), glycolytic inhibition (GI) and oxidative inhibition (OI). It is clearly evident that the holding current was similar at both -80 and -40 mV regardless of metabolic intervention ($n = 5$ for all groups). B, mean $I-V$ relationships recorded 10 min after control conditions, FMI, GI and OI in the presence of 10 μM nifedipine were not significantly different ($n = 5$ for all groups).



by 25.0% (-6.4 ± 0.3 pA pF⁻¹ for control *versus* -4.8 ± 0.1 pA pF⁻¹ after 2-deoxyglucose; $n = 8$, $P < 0.05$) at +10 mV. Unlike previous experiments where steady-state current levels were established with 10 min of exposure to 2-deoxyglucose, the inclusion of NaH₂PO₄ slowed the rate of decline of I_{Ca} (data not shown), so that steady state was achieved after 15 min with I_{Ca} being reduced by 38.4% (-6.2 ± 0.1 pA pF⁻¹ for control *versus* -3.8 ± 0.1 pA pF⁻¹ for 2-deoxyglucose; $n = 4$, $P < 0.01$) as shown in Fig. 4B. This degree of I_{Ca} reduction at steady state was quantitatively similar to that observed following application of 2-deoxyglucose in the absence of NaH₂PO₄ (compare Fig. 4B with Fig. 3B).

The mitochondrial and cytosolic (i.e. myofibrils) creatine kinase catalyses the transfer of high energy phosphate between ATP, ADP, creatine and creatine phosphate, shuttling ATP from the mitochondria to the cytosol. Therefore, it is possible that during glycolytic inhibition under our conditions (i.e. in the absence of creatine) the

mitochondria were able to synthesize ATP but this ATP was not able to reach the Ca²⁺ channels. To test for this possibility, we conducted experiments in the presence of 20 mM creatine and 3 mM NaH₂PO₄ in the pipette solution and 1.2 mM NaH₂PO₄ in the bath solution. The mean $I-V$ relationships shown in Fig. 4C indicate that I_{Ca} at +10 mV decreased by 41.8% from -6.7 ± 0.3 pA pF⁻¹ for control to -3.9 ± 0.6 pA pF⁻¹ ($n = 5$, $P < 0.01$) following 10 min of glycolytic inhibition with creatine and inorganic phosphate. This result was similar to that observed in the absence of creatine and inorganic phosphate, establishing that the mitochondria are unable to sustain I_{Ca} when glycolysis is inhibited.

Given the significant reduction of I_{Ca} following application of 2-deoxyglucose, we sought to use another inhibitor of glycolysis to confirm the role of glycolytic derived ATP in modulating I_{Ca} . The mean $I-V$ relationships in Fig. 4D demonstrate that the application of 100 μM IAA for 10 min caused I_{Ca} to decrease by 44.8% (-6.7 ± 0.3 pA pF⁻¹ for

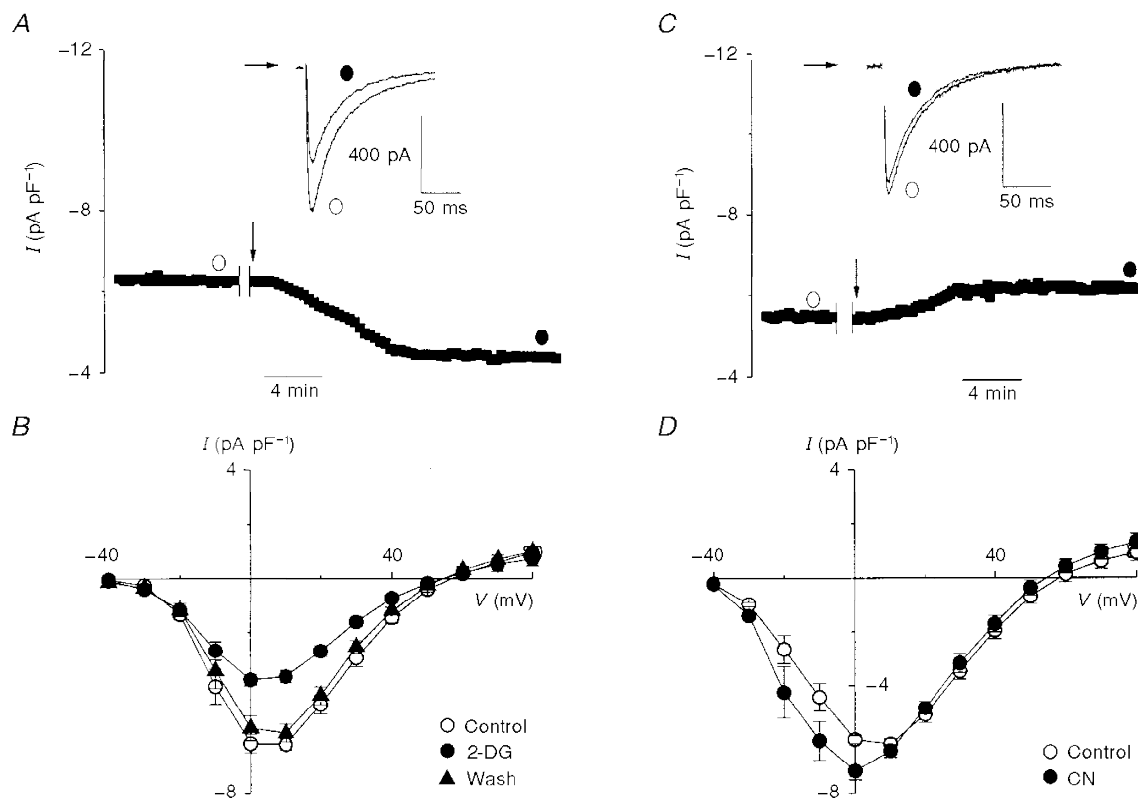


Figure 3. Effect of glycolytic and oxidative inhibition on L-type Ca²⁺ current

A and C, in the whole-cell configuration, I_{Ca} was initially sampled for 5 min prior to the indicated interruption, in order to record a control $I-V$ relationship. This was immediately followed by external wash-in (arrow) of 2-deoxyglucose (2-DG; 10 mM; A) or sodium cyanide (CN; 2 mM; C). The raw traces in A and C (insets) indicate that I_{Ca} was larger prior to (open circle) than after (filled circle) glycolytic inhibition and was not altered by oxidative phosphorylation, respectively. B and D, mean $I-V$ relationships indicating that, on average, 10 min of glycolytic inhibition resulted in a 43% reduction of I_{Ca} at +10 mV ($n = 11$, $P < 0.01$) and was completely reversed by 30 min wash-out of 2-deoxyglucose ($n = 6$) (B). On the other hand, 10 min of oxidative inhibition ($n = 5$) increased I_{Ca} by 16% at 0 mV ($P < 0.05$) and did not alter I_{Ca} at +10 mV and more positive membrane potentials (D).

control *versus* -3.7 ± 0.2 pA pF⁻¹ after application of IAA; $n = 7$, $P < 0.01$) at +10 mV. The similarity in the degree of reduction of I_{Ca} with 2-deoxyglucose and IAA suggests that the reduction of I_{Ca} is indeed the result of glycolytic inhibition (compare Fig. 3B with 4D). The effects of IAA on I_{Ca} were not due to direct effects of IAA on Ca²⁺ channels since, as illustrated in Fig. 4E, the inclusion of 5 mM MgATP in the pipette solution largely prevented the reduction of I_{Ca} by IAA (12.5% reduction of I_{Ca} from -6.4 ± 0.1 pA pF⁻¹ for control to -5.6 ± 0.2 pA pF⁻¹ at +10 mV for IAA-MgATP; $n = 6$, $P < 0.05$).

Previous studies have shown that I_{Ca} is sensitive to pH (Chen *et al.* 1996; Schuhmann *et al.* 1997) and that changes in intracellular pH can occur following the application of 2-deoxyglucose and cyanide (Allue *et al.* 1996). Therefore, to determine whether the reduction of I_{Ca} observed following metabolic inhibition was due to changes in pH, we conducted FMI experiments with increased proton buffering conditions. Figure 5A shows that, after a 10 min FMI period in the presence of 50 mM Hepes in the bath and

pipette solutions, I_{Ca} decreased by almost twofold, similar to the reduction observed after FMI with only 10 mM Hepes (see Fig. 1C and D). The mean $I-V$ relationships shown in Fig. 5B demonstrate that following a 10 min period of FMI in the presence of 50 mM Hepes I_{Ca} at +10 mV decreased by 47.6% (-6.2 ± 0.1 pA pF⁻¹ for control *versus* -3.2 ± 0.2 pA pF⁻¹ after FMI; $n = 6$, $P < 0.01$). Since HCO₃⁻ is a more physiological buffer than Hepes and since Allue *et al.* (1996) successfully clamped pH during metabolic inhibition in isolated myocytes with HCO₃⁻, we conducted a set of experiments with 50 mM Hepes plus 25 mM NaHCO₃ in the bath solution. The NaHCO₃ solution was continuously bubbled with a mixture of 95% O₂ and 5% CO₂ (pH 7.4). The representative myocyte shown in Fig. 5C illustrates that 10 min of FMI with high Hepes and HCO₃⁻ still decreased I_{Ca} . The mean $I-V$ relationships in Fig. 5D show that 10 min of FMI reduced I_{Ca} at +10 mV on average by 38.6%, from -6.4 ± 0.1 pA pF⁻¹ for control to -3.9 ± 0.2 pA pF⁻¹ after FMI in the presence of high Hepes and HCO₃⁻ ($n = 4$, $P < 0.01$). This result was also

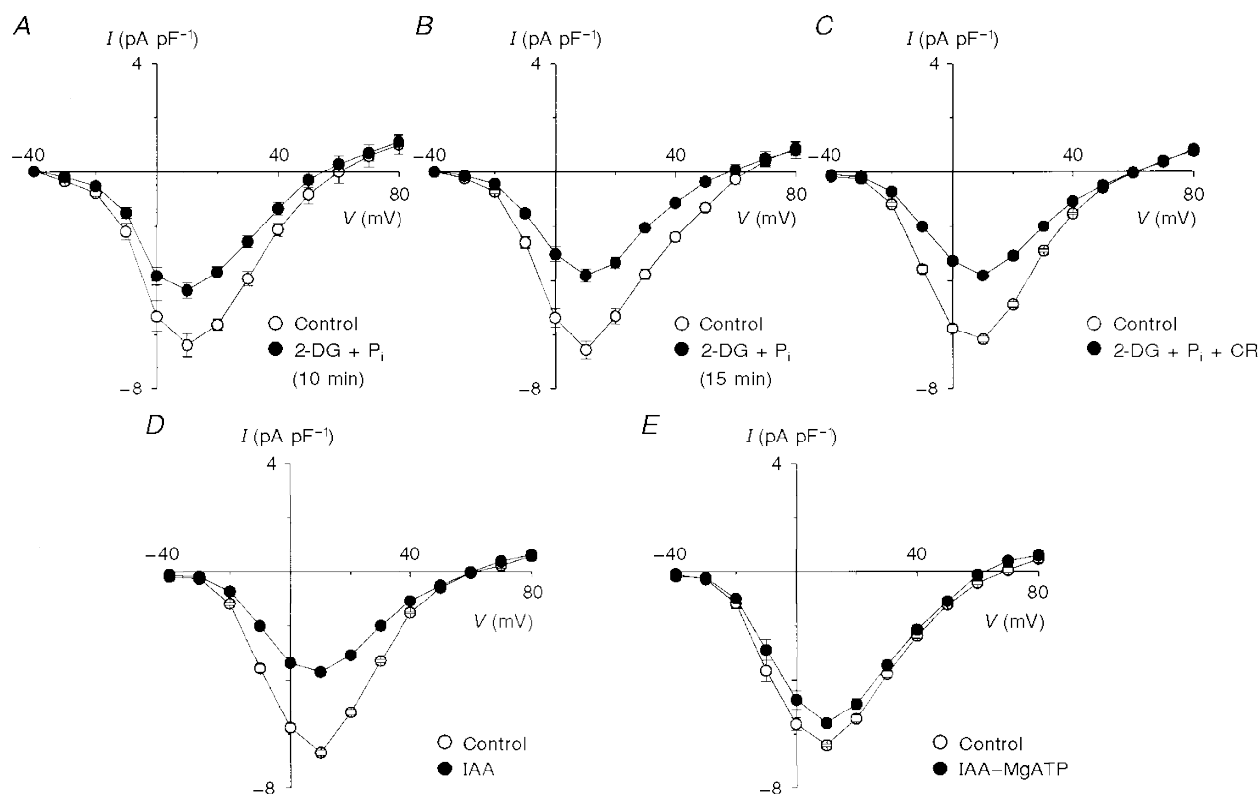


Figure 4. Effect of glycolytic inhibition on Ca²⁺ current with 2-deoxyglucose or iodoacetamide

A, a 10 min episode of glycolytic inhibition with 2-deoxyglucose (2-DG; 10 mM) in the presence of NaH₂PO₄ (P_i; 1.2 mM in bath and 3.0 mM in pipette) reduced I_{Ca} by 25.0% ($n = 4$, $P < 0.05$). B, however, a 15 min exposure to 2-deoxyglucose in the presence of NaH₂PO₄ (1.2 mM in bath and 3.0 mM in pipette) resulted in a greater reduction of I_{Ca} (38.4%, $n = 4$, $P < 0.01$). C, a functional creatine kinase shuttle (20 mM creatine (CR) in pipette) did not prevent reduction of I_{Ca} . D, when myocytes were exposed for 10 min to iodoacetamide (IAA; 100 μ M) a 44.8% reduction of I_{Ca} was observed ($n = 7$, $P < 0.01$), which was similar to the reduction observed following glycolytic inhibition by 2-deoxyglucose (Fig. 3B). E, reduction of I_{Ca} by IAA was greatly attenuated by the inclusion of 5 mM MgATP in the pipette solution (IAA-MgATP), as was also observed when 2-deoxyglucose was used to inhibit glycolysis.

similar to the reduction of I_{Ca} observed during FMI with 10 mM HEPES (Fig. 1D).

Full metabolic inhibition in the presence of exogenous ATP or ATP analogue

In order to test whether the effects of full metabolic or glycolytic inhibition on I_{Ca} resulted from depletion of intracellular ATP, as opposed to other effects of metabolic inhibition, we repeated the full metabolic and glycolytic inhibition experiments but included either ATP or AMP-PCP (a non-hydrolysable analogue of ATP) in the pipette solution. Figure 6A shows typical results when the pipette solution contained MgATP (i.e. 5 mM ATP together with 6.2 mM total MgCl₂). After superfusion with a bath solution containing 2-deoxyglucose and sodium cyanide (arrow) for 10 min, I_{Ca} was reduced by 16% (see also Fig. 6A, inset) without change in the time course of current inactivation (i.e. $t_{1/2} = 33.0 \pm 1.3$ ms in control *versus* 31.4 ± 1.4 ms

following FMI; $n = 4$, n.s.). The mean $I-V$ relationships in Fig. 6B show that I_{Ca} decreased by 18.4% (-6.8 ± 0.6 *versus* -5.5 ± 0.3 pA pF⁻¹, $n = 5$, $P < 0.05$) at +10 mV, compared with a 40% reduction in the absence of ATP in the pipette (Fig. 1D). Associated with this modest reduction of I_{Ca} with ATP, the mean maximum slope conductance was also reduced by 15% from 163 to 142 pS.

Intracellular ATP regulates Ca²⁺ channel activity by phosphorylation-dependent (Hilgemann, 1997) and phosphorylation-independent (O'Rourke *et al.* 1992; Yazawa *et al.* 1997; Yokoshiki *et al.* 1997) pathways. In order to distinguish between these regulatory pathways, experiments were also performed with the non-hydrolysable ATP analogue, AMP-PCP, in the pipette solution. Figure 6C shows that 5 mM MgAMP-PCP (i.e. 5 mM AMP-PCP plus 6.2 mM total MgCl₂) in the patch pipette completely prevented the reduction of I_{Ca} associated with FMI. With

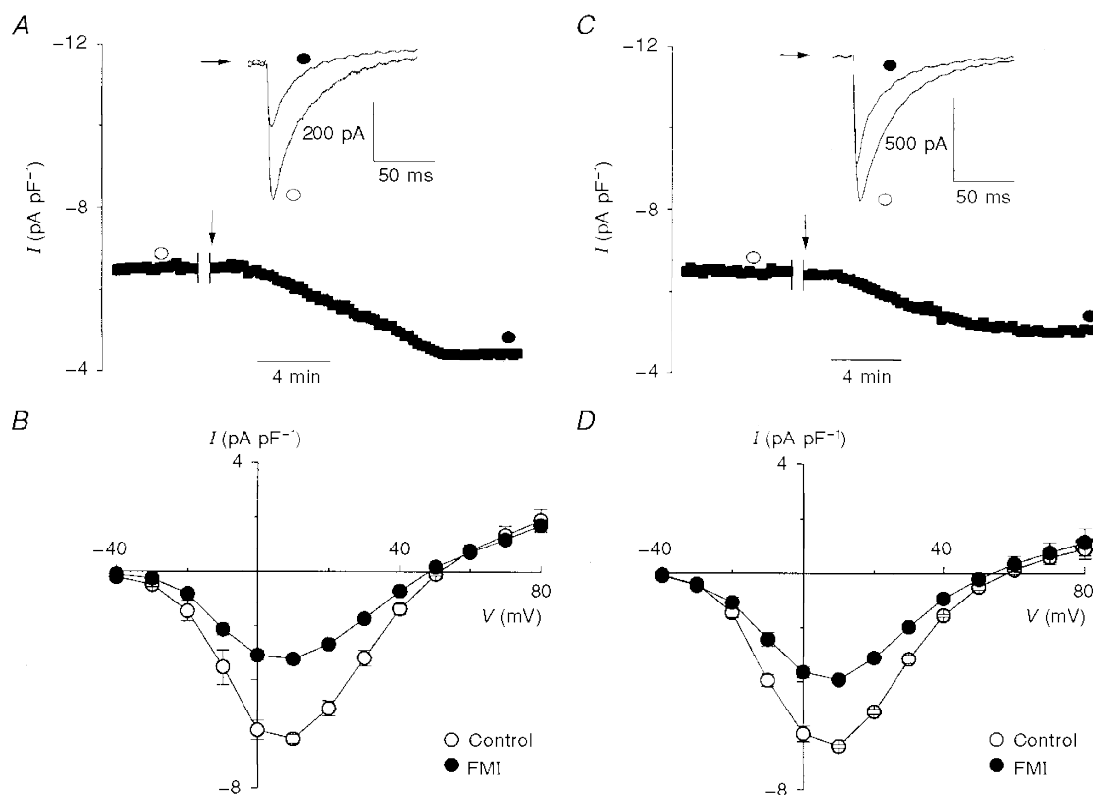


Figure 5. Effect of full metabolic inhibition on Ca²⁺ current in the presence of high HEPES and/or NaHCO₃

To determine whether the reduction of I_{Ca} after FMI is related to alterations in pH_i, proton buffering conditions were improved; 50 mM HEPES was present in the bath and pipette solutions in A–D, plus 25 mM NaHCO₃ in the bath solution in C and D. A and C, in the whole-cell configuration, I_{Ca} was initially sampled for 5 min before the indicated interruption to record a control $I-V$ relationship. This was followed with external wash-in (arrow) of 2-deoxyglucose (10 mM) and sodium cyanide (2 mM) (FMI). In A, it is evident that 50 mM HEPES in the bath and pipette solution did not prevent the reduction of I_{Ca} upon wash-in of metabolic inhibitors. C shows that 50 mM HEPES in the bath and pipette and 25 mM NaHCO₃ in the bath did not prevent the reduction of I_{Ca} upon wash-in of metabolic inhibitors. B and D, mean $I-V$ relationships showing that, on average, metabolic inhibition with 50 mM HEPES only resulted in a 47.6% reduction of I_{Ca} (B; $n = 6$, $P < 0.01$) compared with a 38.6% reduction with 50 mM HEPES and 25 mM NaHCO₃ (D; $n = 4$, $P < 0.01$).

MgAMP-PCP in the pipette, the mean $I-V$ relationships illustrated in Fig. 6D were unaffected by FMI. The peak I_{Ca} at +10 mV was not significantly altered by FMI (-6.9 ± 0.3 pA pF $^{-1}$ in control *versus* -6.5 ± 0.3 pA pF $^{-1}$ following FMI; $n = 5$, n.s.). Similar to MgATP, inclusion of MgAMP-PCP in the pipette had little effect on the maximum slope conductance (194 pS in control *versus* 191 pS following FMI; $n = 5$) and rate of current inactivation ($t_{1/2} = 43.7 \pm 4.5$ ms in control *versus* 41.4 ± 4.1 ms following FMI; $n = 5$, n.s.).

DISCUSSION

The present study shows that a 10 min period of FMI with 2-deoxyglucose and sodium cyanide reduced peak I_{Ca} at +10 mV by 40%, while inhibition of glycolysis alone resulted in a comparable 43% decrease in peak I_{Ca} . On the other hand, inhibition of oxidative phosphorylation alone using cyanide did not have a significant effect on peak I_{Ca} , suggesting that glycolytic derived ATP preferentially

regulates I_{Ca} . This reduction of I_{Ca} by 2-deoxyglucose did not result from non-specific sequestration of inorganic phosphate since inclusion of NaH_2PO_4 could not prevent the inhibitory effects of this non-hydrolysable glucose analogue. This result is consistent with a previous finding in isolated rat hearts where 2-deoxyglucose reduced ATP levels without affecting inorganic phosphate concentration (Kupriyanov *et al.* 1991). The rate of I_{Ca} decline was altered, however, when NaH_2PO_4 was included suggesting that the levels of phosphate may influence the kinetics of I_{Ca} inhibition.

To further establish the role of glycolytic derived ATP in the regulation of I_{Ca} , we also blocked glycolysis with 100 μM IAA, which at this low concentration is a relatively specific inhibitor of glyceraldehyde-3-phosphate dehydrogenase (Sabri & Ochs, 1971). However, unlike 2-deoxyglucose, IAA should not create a sink for inorganic phosphate. Nevertheless, IAA significantly reduced I_{Ca} at +10 mV, similar to 2-deoxyglucose. Taken together these data suggest that substrate metabolism by the mitochondria is unable to maintain I_{Ca} . However, the mitochondrial creatine

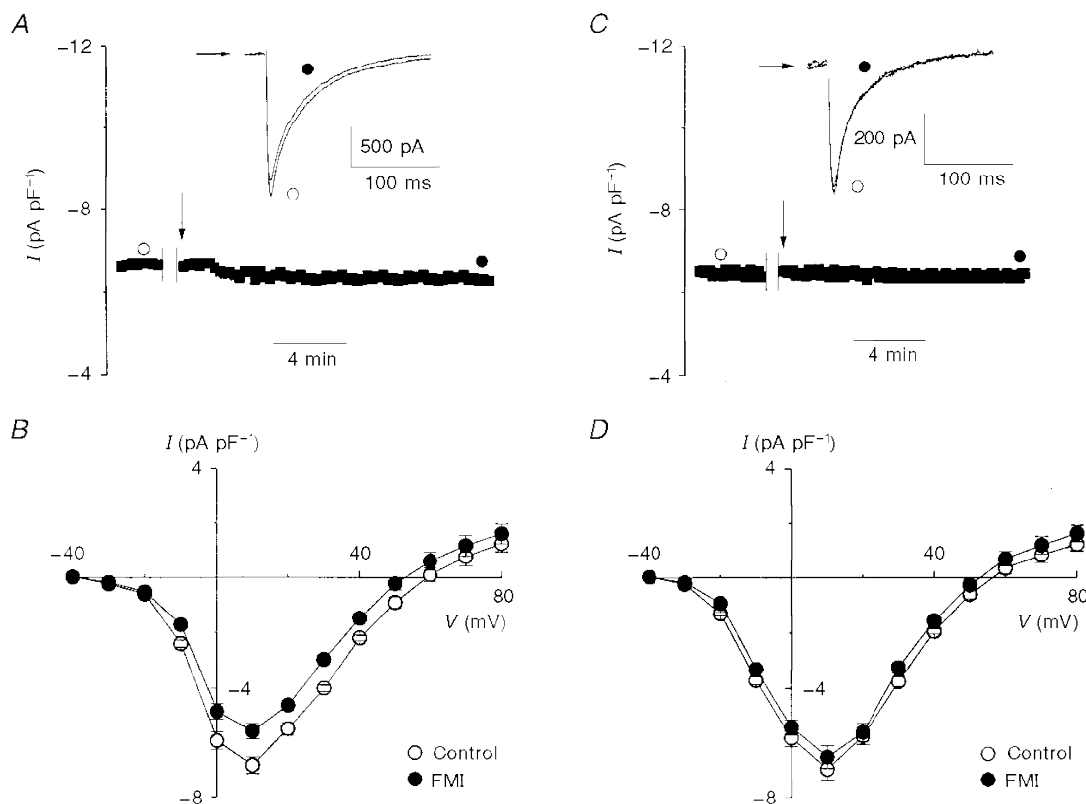


Figure 6. Effect of full metabolic inhibition on L-type calcium current in the presence of MgATP or MgAMP-PCP

MgATP (5 mM) or MgAMP-PCP (5 mM) was included in the pipette solution in A and B, and C and D, respectively. A and C, in the whole-cell configuration, I_{Ca} was initially sampled for 5 min before the indicated interruption in order to record a control $I-V$ relationship. This was immediately followed by external wash-in (arrow) of 2-deoxyglucose (10 mM) and sodium cyanide (2 mM). The raw traces (insets) demonstrate that in the presence of MgATP (A) I_{Ca} prior to (open circle) was larger than I_{Ca} after (filled circle) FMI. However, when MgAMP-PCP was included in the pipette (C) FMI did not alter I_{Ca} . B and D, mean $I-V$ relationships which show that, on average, 10 min of FMI resulted in a moderate but significant reduction of I_{Ca} at +10 mV with MgATP (B; $n = 5$, $P < 0.05$) and was unaltered with MgAMP-PCP (D; $n = 5$, n.s.).

kinase is required to shuttle energy from the mitochondria to the cytosol (Aliev & Saks, 1997). Therefore, it is possible that, when creatine kinase is functional, the mitochondria could prevent I_{Ca} reduction during glycolytic inhibition. However, even in the presence of creatine and phosphate the application of 2-deoxyglucose still caused a significant decrease in I_{Ca} . These results establish that glycolysis and not oxidative phosphorylation is involved in the ATP-dependent reduction of I_{Ca} . The above results are consistent with preferential regulation of cardiac I_{Ca} by glycolysis as previously observed in rat portal vein smooth muscle cells (Lorenz & Paul, 1997). Our results are also consistent with the lack of effect of 1 mM cyanide on I_{Ca} measured in feline cardiomyocytes (Furukawa *et al.* 1994). Preferential regulation of cardiac L-type Ca^{2+} channels by glycolytic ATP is also expected from the sensitivity of other cardiac membrane proteins to glycolytic inhibition (Weiss & Lamp, 1987, 1989; Xu *et al.* 1995).

Since biphasic changes in intracellular pH occur following metabolic inhibition with cyanide and 2-deoxyglucose and since protons modulate I_{Ca} , we tested whether increasing intracellular pH buffering capacity with the addition of 50 mM Hepes and 25 mM $NaHCO_3$ would affect the changes in I_{Ca} observed with metabolic inhibition. Increasing the pH buffering capacity with high levels of HCO_3^- , previously shown to prevent changes in intracellular pH (Allue *et al.* 1996), had no effect on the magnitude or rate of I_{Ca} reduction following metabolic inhibition. Thus, the changes in I_{Ca} are unlikely to be mediated by changes in intracellular pH.

Prevention of I_{Ca} reduction following metabolic inhibition by MgATP or MgAMP-PCP in the pipette solution (Fig. 6B and D; cf. Fig. 1D) suggests that metabolic regulation by glycolysis involves direct allosteric phosphorylation-independent effects of ATP on L-type Ca^{2+} channels. These observations are consistent with previous studies in guinea-pig myocytes (O'Rourke *et al.* 1992; Yazawa *et al.* 1997) and vascular smooth muscle cells (Yokoshiki *et al.* 1997) where it was shown that Mg^{2+} must be complexed with ATP or its analogues (AMP-PCP or AMP-PNP (adenylyl imidophosphate)) to modulate I_{Ca} . We found that MgAMP-PCP completely prevented the reduction of I_{Ca} associated with FMI.

The more potent effect of MgAMP-PCP compared with MgATP (compare Fig. 6B with Fig. 6D) in preventing I_{Ca} reduction during FMI might be related to local ATP concentrations. With ATP in the pipette, local ATP concentrations could be reduced by cellular ATPases, which utilize ATP at a rate comparable to the rate of ATP diffusion into the cell from the pipette. This local consumption would not be expected for non-hydrolysable analogues of ATP, like AMP-PCP. In addition, local cellular ATPases may enhance the intracellular ADP concentration locally and thereby compete with ATP for the allosteric binding site, as has been reported for the ATP-sensitive K^+ channel (Terzic *et al.* 1995) and CFTR (cystic fibrosis transmembrane

conductance regulator) Cl^- channel (Anderson & Welsh, 1992; Gadsby *et al.* 1995).

In this study the effect of FMI on intracellular ATP concentration was not measured. However, depletion of the global intracellular ATP concentration to below 20 μM is associated with rigor cross-bridge formation and square-shaped cell morphology (Fabiato & Fabiato, 1975; Allue *et al.* 1996). In our studies, when ATP was not included in the pipette, FMI and inhibition of oxidative phosphorylation consistently caused cells to shorten by 50–60% and become square shaped within 3–5 min of exposure to the inhibitors, suggesting that the intracellular ATP concentration was reduced to less than 20 μM (Allue *et al.* 1996). However, inhibition of glycolysis and inclusion of MgATP or MgAMP-PCP during FMI was not associated with cell shortening, suggesting that a global reduction of ATP to less than 20 μM did not occur and was not required for I_{Ca} reduction. These results are expected since contractile proteins have been previously shown to be preferentially supported by oxidative phosphorylation (Weiss & Lamp, 1987, 1989).

It is conceivable that the reduction of I_{Ca} during FMI is related to the substantial changes in myocyte shape that occur as a result of rigor cross-bridge development. For instance, cell shrinkage as a result of hyperosmotic perfusion has been shown to decrease, while cell swelling has been shown to increase, peak Ba^{2+} currents through Ca^{2+} channels in smooth muscle (Xu *et al.* 1996). Similarly, cell shrinkage reduces the amplitude of I_{Ca} and slows its rate of inactivation in guinea-pig ventricular myocytes, secondary to rapid elevation of intracellular Ca^{2+} (Ogura *et al.* 1997). On the other hand, it has also been demonstrated in guinea-pig cardiomyocytes that I_{Ca} is not altered by 20% stretch or hyposmotic stress (Sasaki *et al.* 1992) in rat cardiomyocytes (Hongo *et al.* 1996). In our study it is unlikely that the reduction of I_{Ca} is due to cell shortening since the reductions of I_{Ca} were observed after glycolytic inhibition (with 2-deoxyglucose or IAA), where cell shortening was not observed. Furthermore, allosteric regulation by MgATP has been shown to occur in the presence of 5 mM intracellular ATP, where rigor and cell shape changes are prevented (O'Rourke *et al.* 1992; Yokoshiki *et al.* 1997).

In summary, we have shown that cardiac L-type I_{Ca} is preferentially regulated by MgATP derived from glycolysis as opposed to oxidative phosphorylation and that regulation by ATP does not require hydrolysis. This preferential utilization of glycolytic ATP by L-type Ca^{2+} channels could reflect the observation that glycolytic enzymes are colocalized with skeletal Ca^{2+} channels (Brandt *et al.* 1990), similar to A-kinase anchoring proteins (Goa *et al.* 1997). This regulatory mechanism may be extremely important under both physiological and pathophysiological conditions. Under physiological conditions this phosphorylation-independent mechanism may operate separate from the phosphorylation-dependent mechanisms. It has been demonstrated that the K_d for 50% potentiation of L-type Ca^{2+} channel activity by internal perfusion of 5 mM ATP is

0.53 mM (Yokoshiki *et al.* 1997), which is much higher than the K_d (i.e. 15 μ M) for phosphorylation (Francis & Corbin, 1994; Hilgemann, 1997). Therefore, this phosphorylation-independent pathway represents an important regulatory control mechanism for I_{Ca} activity operating at relatively high levels of intracellular ATP. This regulatory mechanism of I_{Ca} would be expected to work in concert with a shortening of the action potential duration to reduce excitability and contractility, thereby conserving energy during metabolic stress.

- ALIEV, M. K. & SAKS, V. A. (1997). Compartmentalized energy transfer in cardiomyocytes: use of mathematical modeling for analysis of in vivo regulation of respiration. *Biophysical Journal* **73**, 428–445.
- ALLUE, I., GANDELMAN, O., DEMENTIEVA, E., UGAROVA, N. & COBBOLD, P. (1996). Evidence for rapid consumption of millimolar concentrations of cytoplasmic ATP during rigor-contraction of metabolically compromised single cardiomyocytes. *Biochemical Journal* **319**, 463–469.
- ANDERSON, M. P. & WELSH, M. J. (1992). Regulation by ATP and ADP of CFTR chloride channels that contain mutant nucleotide-binding domains. *Science* **257**, 1701–1704.
- BACKX, P. H. & MARBAN, E. (1993). Background potassium current active during the plateau of the action potential in guinea pig ventricular myocytes. *Circulation Research* **72**, 890–900.
- BACKX, P. H., O'ROURKE, B. & MARBAN, E. (1991). Flash photolysis of magnesium-DM-nitrophen in heart cells: a novel approach to probe magnesium- and ATP-dependent regulation of calcium channels. *American Journal of Hypertension* **4**, 416–421S.
- BRANDT, N. R., CASWELL, A. H., WEN, S. R. & TALVENHEIMO, J. A. (1990). Molecular interactions of the junctional foot protein and dihydropyridine receptor in skeletal muscle triads. *Journal of Membrane Biology* **113**, 237–251.
- CAMPBELL, J. D. & PAUL, R. J. (1992). The nature of fuel provision for the Na^+,K^+ -ATPase in porcine vascular smooth muscle. *Journal of Physiology* **447**, 67–82.
- CARABAZA, A., CIUDAD, C. J., BAQUE, S. & GUINOVART, J. J. (1992). Glucose has to be phosphorylated to activate glycogen synthase, but not to inactivate glycogen phosphorylase in hepatocytes. *FEBS Letters* **296**, 211–214.
- CHEN, X. H., BEZPROZVANNY, I. & TSIEN, R. W. (1996). Molecular basis of proton block of L-type Ca^{2+} channels. *Journal of General Physiology* **180**, 363–374.
- CRITZ, S. D., LIU, G. S., CHUJO, M. & DOWNEY, J. M. (1997). Pinacidil but not nicorandil opens ATP-sensitive K^+ channels and protects against simulated ischemia in rabbit myocytes. *Journal of Molecular and Cellular Cardiology* **29**, 1123–1130.
- DEUTSCH, N., KLITZNER, T. S., LAMP, S. T. & WEISS, J. N. (1991). Activation of cardiac ATP-sensitive K^+ current during hypoxia: correlation with tissue ATP levels. *American Journal of Physiology* **261**, H671–676.
- DEUTSCH, N. & WEISS, J. N. (1993). ATP-sensitive K^+ channel modification by metabolic inhibition in isolated guinea-pig ventricular myocytes. *Journal of Physiology* **465**, 163–179.
- DRINGEN, R. & HAMPRECHT, B. (1993). Inhibition by 2-deoxyglucose and 1,5-gluconolactone of glycogen mobilization in astroglia-rich primary cultures. *Journal of Neurochemistry* **60**, 1498–1504.
- FABIATO, A. & FABIATO, F. (1975). Effects of magnesium on contractile activation of skinned cardiac cells. *Journal of Physiology* **249**, 497–517.
- FRANCIS, S. H. & CORBIN, J. D. (1994). Structure and function of cyclic nucleotide-dependent protein kinases. *Annual Review of Physiology* **56**, 237–272.
- FURUKAWA, T., MYERBURG, R. J., FURUKAWA, N., KIMURA, S. & BASSETT, A. L. (1994). Metabolic inhibition of $I_{Ca,L}$ and I_K differs in feline left ventricular hypertrophy. *American Journal of Physiology* **266**, H1121–1131.
- GADSBY, D. C., NAGEL, G. & HWANG, T. C. (1995). The CFTR chloride channel of mammalian heart. *Annual Review of Physiology* **57**, 387–416.
- GOA, T., YATANI, A., DELL'ACQUA, M. L., SAKO, H., GREEN, S. A., DASCAL, N., SCOTT, J. D. & HOSEY, M. M. (1997). cAMP-dependent regulation of cardiac L-type Ca^{2+} channels requires membrane targeting of PKA and phosphorylation of channel subunits. *Neuron* **19**, 185–196.
- HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.
- HARDIN, C. D., RAEYMAEKERS, L. & PAUL, R. J. (1992). Comparison of endogenous and exogenous sources of ATP in fueling Ca^{2+} uptake in smooth muscle plasma membrane vesicles. *Journal of General Physiology* **99**, 21–40.
- HAZEN, S. L., WOLF, M. J., FORD, D. A. & GROSS, R. W. (1994). The rapid and reversible association of phosphofructokinase with myocardial membranes during myocardial ischemia. *FEBS Letters* **339**, 213–216.
- HILGEMANN, D. W. (1997). Cytoplasmic ATP-dependent regulation of ion transporters and channels: mechanisms and messengers. *Annual Review of Physiology* **59**, 193–220.
- HONGO, K., WHITE, E., LE GUENNEC, J.-Y. & ORCHARD, C. H. (1996). Changes in $[Ca^{2+}]_i$, $[Na^+]_i$ and Ca^{2+} current in isolated rat ventricular myocytes following an increase in cell length. *Journal of Physiology* **491**, 609–619.
- IRISAWA, H. & KOKUBUN, S. (1983). Modulation by intracellular ATP and cyclic AMP of the slow inward current in isolated single ventricular cells of the guinea-pig. *Journal of Physiology* **338**, 321–337.
- KUPRIYANOV, V. V., LAKOMKIN, V. L., KORCHAZHKINA, O. V., STEINSCHEIDER, A. YA., KAPELKO, V. I. & SAKS, V. A. (1991). Control of cardiac energy turnover by cytoplasmic phosphates: ^{31}P -NMR study. *American Journal of Physiology*, suppl., **261**, 45–53.
- LORENZ, J. N. & PAUL, R. J. (1997). Dependence of Ca^{2+} channel currents on endogenous and exogenous sources of ATP in portal vein smooth muscle. *American Journal of Physiology* **272**, H987–994.
- LYNCH, R. M. & BALABAN, R. S. (1987). Coupling of aerobic glycolysis and Na^+-K^+ -ATPase in renal cell line MDCK. *American Journal of Physiology* **252**, C269–276.
- LYNCH, R. M. & PAUL, R. J. (1987). Compartmentation of carbohydrate metabolism in vascular smooth muscle. *American Journal of Physiology* **252**, C328–334.
- OGURA, T., YOU, Y. & McDONALD, T. F. (1997). Membrane currents underlying the modified electrical activity of guinea-pig ventricular myocytes exposed to hyperosmotic solution. *Journal of Physiology* **504**, 135–151.

- OIKONOMAKOS, N. G., ZOGRAPHOS, S. E., JOHNSON, L. N., PAPAGEORGIOU, A. C. & ACHARYA, K. R. (1995). The binding of 2-deoxy-D-glucose 6-phosphate to glycogen phosphorylase b: kinetic and crystallographic studies. *Journal of Molecular Biology* **254**, 900–917.
- O'ROURKE, B., BACKX, P. H. & MARBAN, E. (1992). Phosphorylation-independent modulation of L-type calcium channels by magnesium–nucleotide complexes. *Science* **257**, 245–248.
- PAUL, R. J., HARDIN, C. D., RAEYMAEKERS, L., WUYTACK, F. & CASTEELS, R. (1989). Preferential support of Ca²⁺ uptake in smooth muscle plasma membrane vesicles by an endogenous glycolytic cascade. *FASEB Journal* **3**, 2298–2301.
- PIERCE, G. N. & PHILIPSON, K. D. (1985). Binding of glycolytic enzymes to cardiac sarcolemmal and sarcoplasmic reticular membranes. *Journal of Biological Chemistry* **260**, 6862–6870.
- POPE, A. J., RICHARDSON, S. K., IFE, R. J. & KEELING, D. J. (1991). Characterisation of the effects of anthranilic and (indanyloxy) acetic acid derivatives on chloride transport in membrane vesicles. *Biochimica et Biophysica Acta* **1067**, 51–63.
- REUTER, H. & SCHOLTZ, H. (1977). The regulation of the calcium conductance of cardiac muscle by adrenaline. *Journal of Physiology* **264**, 49–62.
- SABRI, M. I. & OCHS, S. (1971). Inhibition of glyceraldehyde-3-phosphate dehydrogenase in mammalian nerve by iodoacetic acid. *Journal of Neurochemistry* **18**, 1509–1514.
- SASAKI, N., MITSUIYE, T. & NOMA, A. (1992). Effects of mechanical stretch on membrane currents of single ventricular myocytes of guinea-pig heart. *Japanese Journal of Physiology* **42**, 957–970.
- SCHOENMAKERS, T. J. M., VISSER, G. J., FLIK, G. & THEUVENET, A. P. R. (1992). CHELATOR: An improved method for computing metal ion concentrations in physiological solutions. *Biotechniques* **12**, 870–874.
- SCHUHMAN, K., VOELKER, C., HOFER, G. F., PFLUGELMEIER, H., KLUGBAUER, N., HOFMANN, F., ROMANIN, C. & GROSCHNER, K. (1997). Essential role of the beta subunit in modulation of C-class L-type Ca²⁺ channels by intracellular pH. *FEBS Letters* **408**, 75–80.
- TANIGUCHI, J., NOMA, A. & IRISAWA, H. (1983). Modification of the cardiac action potential by intracellular injection of adenosine triphosphate and related substances in guinea pig single ventricular cells. *Circulation Research* **53**, 131–139.
- TERZIC, A., JAHANGIR, A. & KURACHI, Y. (1995). Cardiac ATP-sensitive K⁺ channels: regulation by intracellular nucleotides and K⁺ channel-opening drugs. *American Journal of Physiology* **269**, C525–545.
- WEISS, J. & HILTBRAND, B. (1985). Functional compartmentation of glycolytic versus oxidative metabolism in isolated rabbit heart. *Journal of Clinical Investigation* **75**, 436–447.
- WEISS, J. N. & LAMP, S. T. (1987). Glycolysis preferentially inhibits ATP-sensitive K⁺ channels in isolated guinea pig cardiac myocytes. *Science* **238**, 67–69.
- WEISS, J. N. & LAMP, S. T. (1989). Cardiac ATP-sensitive K⁺ channels: Evidence for preferential regulation by glycolysis. *Journal of General Physiology* **94**, 911–935.
- WEISS, J. N., VENKATESH, N. & LAMP, S. T. (1992). ATP-sensitive K⁺ channels and cellular K⁺ loss in hypoxic and ischaemic mammalian ventricle. *Journal of Physiology* **447**, 649–673.
- XU, K. Y., ZWEIER, J. L. & BECKER, L. C. (1995). Functional coupling between glycolysis and sarcoplasmic reticulum Ca²⁺ transport. *Circulation Research* **77**, 88–97.
- XU, W. X., KIM, S. J., KIM, S. J., SO, I., KANG, T. M., RHEE, J. C. & KIM, K. W. (1996). Effect of stretch on calcium channel currents recorded from the antral circular myocytes of guinea-pig stomach. *Pflügers Archiv* **432**, 159–164.
- YAZAWA, K., KAMEYAMA, A., YASUI, K., LI, J.-M. & KAMEYAMA, M. (1997). ATP regulates cardiac Ca²⁺ channel activity via a mechanism independent of protein phosphorylation. *Pflügers Archiv* **433**, 557–562.
- YOKOSHIKI, H., KATSUBE, Y. & SPERELAKIS, N. (1997). Regulation of Ca²⁺ channel currents by intracellular ATP in smooth muscle cells of rat mesenteric artery. *American Journal of Physiology* **272**, H814–819.

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Corresponding author

P. H. Backx: Center for Cardiovascular Research, Toronto General Hospital, CCRW 3-802, 101 College Street, Toronto, Ontario, Canada M5G 1L7.

Email: p.backx@utoronto.ca