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Opening of Cardiac Sarcolemmal K_{ATP} Channels by Dinitrophenol Separate from Metabolic Inhibition

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Abstract. Opening of ATP-sensitive K^+ (K_{ATP}) channels by the uncoupler of oxidative phosphorylation, 2,4 dinitrophenol (DNP), has been assumed to be secondary to metabolic inhibition and reduced intracellular ATP levels. Herein, we present data which show that DNP (200 μM) can induce opening of cardiac K_{ATP} channels, under whole-cell and inside-out conditions, despite millimolar concentrations of ATP (1–2.5 mM). DNP-induced currents had a single channel conductance (71 pS), inward rectification, reversal potential, and intraburst kinetic properties (open time constant, τ_{open}: 4.8 msec; fast closed time constant, $\tau_{closed(f)}$: 0.33 msec) characteristic of K_{ATP} channels suggesting that DNP did not affect the pore region of the channel, but may have altered the functional coupling of the ATP-dependent channel gating. A DNP analogue, with the pH-titrable hydroxyl replaced by a methyl group, could not open K_{ATP} channels. The pH-dependence of the effect of DNP on channel opening under whole-cell, cell-attached, and inside-out conditions suggested that transfer of protonated DNP across the sarcolemma is essential for activation of K_{ATP} channels in the presence of ATP. We conclude that the use of DNP for metabolic stress-induced K_{ATP} channel opening should be reevaluated.

Key words: ATP-sensitive K^+ channels — Uncoupler of oxidative phosphorylation — Protonophore — pH — Cardiac — DNP

Introduction

The ATP-sensitive potassium (K_{ATP}) channel is considered to be a sensor of the metabolic state of a myocardial cell, and activation of these channels an indicator of cellular metabolic compromise (Aschroft & Ashcroft, 1990; Nichols & Lederer, 1991; Takano & Noma, 1993; Findlay, 1994; Lazdunski, 1994). Metabolic poisons, which include the weak acid 2,4-dinitrophenol (DNP) an uncoupler of oxidative phosphorylation (Loomis & Lippmann, 1948), are commonly used to open K_{ATP} channels, normally kept closed by millimolar levels of intracellular ATP. It has been assumed that DNP opens K_{ATP} channels by inhibiting mitochondrial oxidative phosphorylation so decreasing intracellular levels of ATP (Isenberg et al., 1983; Findlay, 1993; Weiss & Venketash, 1993). However, this conventional assumption has been disputed.

In cardiac cells, DNP can open K_{ATP} channels prior to substantial change in the levels of intracellular nucleotides (Weiss & Hiltbrand 1985; Decking et al., 1995), with minimal effects on other metabolism-sensitive channels, such as the Na^+ channel (Mejia-Alvarez & Marban, 1992; Wu et al., 1992). These findings indicate that DNP-induced activation of K_{ATP} channels may not be exclusively due to cellular metabolic inhibition. Moreover, DNP-induced K_{ATP} channel activity loses its sensitivity to inhibitory channel ligands, such as sulfonylurea drugs (Findlay, 1993; Guillemare, Lazdunski & Honore, 1995), suggesting that DNP may alter the regulatory properties of K_{ATP} channels.

DNP, like other protonophores (McLaughlin, 1972), affects ion fluxes not only at the level of mitochondria, but also at the plasmalemma itself (Johnstone, 1978; Holmuhamedov, Sadykov & Teplova, 1987; Dijkstra et al., 1994). This is of importance since in sarcolemmal membranes intracellular protons and endogenous weak acids do enhance opening of K_{ATP} channels in the presence of intracellular ATP (Koyano et al., 1993; McKillen et al., 1994; Vivaudou & Forestier, 1995). It is, therefore, conceivable that by crossing the plasmalemma, in a pH-dependent manner, DNP may affect K_{ATP} channel behavior even before reaching the mitochondria. Under-

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standing this action of DNP, therefore, may define a pathway of K_{ATP} channel gating independent from depletion of intracellular ATP.

Thus, we studied the action of DNP on K_{ATP} channels exposed to millimolar ATP concentrations at the whole-cell level and in plasmalemmal patches under cell-free conditions. Our findings argue for reconsideration of the conventional assumption that DNP opens K_{ATP} channels solely by depletion of intracellular ATP.

Methods

Cell Isolation

Ventricular myocytes were isolated by enzymatic dissociation (Alekseev et al., 1996*b*). Solutions were prepared based on a ''low-Ca^{2+} medium'' (in mM): NaCl 100, KCl 10, KH_2PO_4 1.2, $MgSO_4$ 5, glucose 20, taurine 50, HEPES 10 (pH 7.2–7.3). Guinea pigs were anesthetized with pentobarbital (1 ml/100 mg of weight i.p.). Following cardiotomy, the heart was retrogradely perfused (at 37°C) with: medium 199 (Sigma) for 2–3 min, followed by Ca^{2+}-EGTA-buffered low Ca^{2+} medium (pCa 7) for 80 sec, and finally low Ca^{2+} medium containing pronase E (8 mg per 100 ml, Serva), proteinase K (1.7 mg per 100 ml, Boehringer Mannheim), bovine albumin) 0.1 g *per* 100 ml, fraction V, Sigma) and 200 μM $CaCl_2$. Ventricles were separated from atria, and cut into small fragments (6–10 mm^3) in the low Ca^{2+} medium enriched with 200 μM $CaCl_2$. Single cells were then isolated by stirring the tissue (at 37°C) in a solution containing pronase E and proteinase K supplemented with collagenase (5 mg *per* 10 ml, Worthington). After 10 min, the first aliquot was removed, filtered through a nylon sieve, centrifuged (at 300–400 rpm), and washed twice. Remaining tissue fragments were reexposed to collagenase, and isolation continued for 2–3 such cycles. Isolated cardiomyocytes were stored in low Ca^{2+} medium with 200 μM $CaCl_2$. Rod-shaped cardiomyocytes with clear striations and smooth surface were used. The membrane potential, measured using the current-clamp mode of the patch clamp technique (Hamill et al., 1981), was within −66 to −70 mV ($n = 26$; Alekseev et al., 1996*b*). Experiments were performed with the approval of the Institutional Animal Care and Use Committee.

Electrophysiological Recordings

The patch-clamp method was employed in the whole-cell, cell-attached, and inside-out configuration (Hamill et al., 1981) at 20–22°C, and measurements made using a patch-clamp amplifier (Axopatch-1C, Axon Instruments). For whole-cell recordings, cardiomyocytes were superfused with Tyrode (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). Whole-cell currents were obtained in response to rectangular or ramp-shape pulses from a holding potential of −50 mV to test potentials, sampled and analyzed with an Everex Step computer using the ''BioQuest'' software (Alekseev et al., 1996*a*). Fire-polished pipettes coated with Sylgard (resistance 5–7 MΩ) were filled with ''internal solution'' (in mM): KCl 140, $MgCl_2$ 1, EGTA 5, HEPES-KOH 5 (pH 7.3) supplemented with 2.5 mM ATP. The rate of ATP exchange between the cytosol and the pipette solution was calculated based on the assumption that increase in the ATP concentration due to diffusion is expressed by an exponential process (Kameyama, Hofmann & Trautwein, 1985):

$$c(t) = c_o\left(1 - \exp\left(-\frac{D \cdot t}{V \cdot G}\right)\right),$$

where $c(t)$ is the time-dependent intracellular concentration; c_o the concentration of ATP in the pipette (2.5 mM); V, cell volume ($1 \cdot 10^{-14}$ m^3); D, diffusion coefficient ($5 \cdot 10^{-10}$ m^2/s); and G, a parameter which depends upon the geometry of the pipette:

$$G = 1/[\pi r_o(r_o + \tan \phi)],$$

where r_O, the radius of the pipette tip (1.4 μm); and ϕ, the half-tip cone angle (8°). For the values used, 10–15 sec following whole-cell formation approximately 1 mM of ATP can be expected to have reached the cytosol, and at 100 sec the estimated concentration would approach the equilibrium concentration of ATP in the cytosol (*see also* Kameyama et al., 1985). Actual exchange may not have been as complete as predicted from the above equation due to the nonspherical form of cardiac cells, and differences in the diffusion constant for a cell interior *vs.* aqueous solution.

In the cell-attached or inside-out configuration, fire-polished and coated pipettes (resistance 8–10 MΩ), were filled with ''pipette solution'' (in mM): KCl 140, $CaCl_2$ 1, $MgCl_2$ 1, HEPES-KOH 5 (pH 7.3), and cells bathed in ''internal solution'' (see above for composition). Pipette potential was +60 mV (i.e., patches were held at –60 mV) unless otherwise indicated. Single-channel recordings were monitored on a high-gain digital storage oscilloscope (VC-6025; Hitachi) and stored on tape using a PCM converter system (VR-10, Instrutech). Data were reproduced, low-pass filtered at 1.5 KHz (−3 dB) by a Bessel filter (Frequency Devices 902), sampled at 4 KHz, and analyzed by the ''BioQuest'' program. The threshold for judging the open state of channels was set at half single channel amplitude. The degree of channel activity was assessed by digitizing segments of current records, and expressed as NP_o, where N represents the number of channels in the patch and P_O the probability of each channel to be open. For further quantitative analysis, NP_o values were plotted in a cumulative manner, and the slope of cumulative NP_o values fitted by linear regression.

Drugs

DNP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), glyburide (Sigma), 2,4-dinitrotoluene (DNT; Fluka), and nigericin (Molecular Probes) were dissolved in dimethylsulfoxide was <0.1%, which did not affect K_{ATP} channels.

Statistics

Results are expressed as mean ± SE; n refers to the number of myocytes used in each analysis. Vertical bars in graphs correspond to SE. Statistical significance was determined by Student's t test, and a value of $P < 0.05$ considered to indicate a significant difference.

Results

Intracellular ATP Does Not Prevent DNP-induced K_{ATP} Current at the Whole Cell Level

In the absence of DNP, no significant K_{ATP} current was observed within the time frame of experiments (Fig. 1*A*,

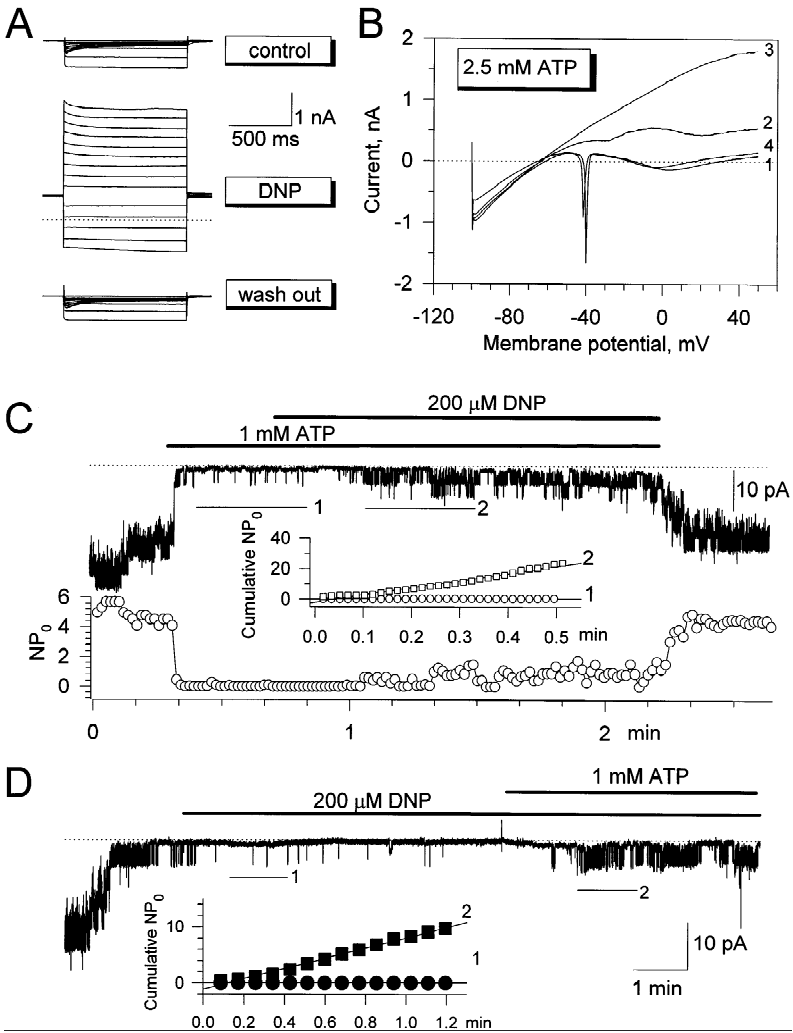

Fig. 1. DNP-induced outward K^+ current in the presence of ATP. (*A* and *B*). Whole-cell voltage clamped cardiomyocytes (*A*) Original current records obtained in the presence of 2.5 mM ATP in the pipette solution in control (upper traces), at the maximal effect of 200 μM DNP (middle traces), and after washout of DNP (lower traces. Holding potential was −50 mV. Rectangular, 1000-msec long, pulses were applied (in 10-mV steps) from −100 to +40 mV every 3 sec. (*B*) Voltage-current relationships, with 2.5 mM ATP present in the pipette solution, obtained by a 0.15 V/s ramp pulse stimulation from −100 to +50 mV. *Trace 1:* control; *traces 2–3:* at 4 and 8 min following addition of DNP (200 μM); *trace 4:* at 7 min following washout of DNP. In trace 1, the first dip (peak at −40 mV) is due to the inward Na^+ current, and the second dip (peak at 0 mV) to the inward Ca^{2+} current; trace 2 indicates a partial effect of DNP on induction of an outward current with concomitant decrease of both Na^+ and Ca^{2+} currents; trace 3 represents the fully developed DNP-induced, moderately inward rectifying, K^+ current; trace 4 indicates almost complete recovery of control current upon washout of DNP. (*C* and *D*) Single-channel records in the inside-out patch-clamp mode. Holding potential: −50 mV. In (*C*) Corresponding NP_0 values calculated over 1-sec periods plotted as function of time. *Insets:* Cumulative NP_0 values, corresponding to segments of original traces (denoted by horizontal bars beneath the original trace), calculated in *C* in the presence of ATP (circles, segment 1), and in the presence of ATP plus DNP (squares, segment 2), and in *D* in the presence of DNP alone (circles, segment 1), and DNP plus ATP (squares, segment 2). Results of linear regression are presented as solid lines, labeled 1 and 2, corresponding to respective segments.

control; n = 43). Control records (Fig. 1*A*) displayed current properties characteristic of ventricular myocytes, with strongly inward-rectifying I_{KI} current at hyperpolarizing potentials and appearance of Na^+ and Ca^{2+} currents with membrane depolarization (Fig. 1*B*, trace 1). DNP (200 μM), added to the bath, induced a prominent current despite the presence of 2.5 mM ATP in the pipette ($n = 7$; Fig. 1*A*). This current was time-independent and outwardly directed, dominant at potentials positive to −50 mV (Fig. 1*B*, traces 2–3), as expected for a current flowing through weakly inward-rectifying K_{ATP} channels. The time required for initiation of the DNP-induced current, and for development of its maximal amplitude (1.27 ± 0.29 nA at 0 mV membrane potential) was 306 ± 65 sec and 571 ± 115 sec ($n = 7$), respectively. Washout of DNP largely restored control currents, including the Na^+ and Ca^{2+} current components (Fig. 1*A* and *B*, trace 4; $n = 24$). Within a single cardiomyocyte ($n = 15$), the DNP-induced outward current was readily reversible and reproducible (2–3 times); following repeated exposure to DNP, the sensitivity of this current to the sulfonylurea, glyburide (6 μM; $n = 6$) was lost (*see also* Findlay, 1993).

DNP-induced K_{ATP} Channel Activity in the Presence of ATP in Excised Membrane Patches

Following patch excision, spontaneous K_{ATP} channel activity was vigorous (NP_0 ~5; Fig. 1*C*). ATP (1 mM) inhibited channel activity, decreasing NP_0 to virtually 0 (slope of cummulative NP_0: 1.1 min^{-1}; Fig. 1*C*). DNP (200 μM) enhanced K_{ATP} channel activity (within 30-sec) despite the presence of ATP (NP_0 ~1.2; slope value: 46.9 min^{-1}; Fig. 1*C*). Following washout of ATP and DNP, K_{ATP} channel activity was partially restored (NP_0 ~4; Fig. 1*C*). On average ($n = 4$), K_{ATP} channel activity measured as the slope of cumulative NP_0 was 0.6 ± 0.3 min^{-1} in ATP alone and 22.2 ± 12.4 min^{-1} in ATP plus DNP ($P < 0.05$; Fig. 2*A*). Similarly, after partial run-

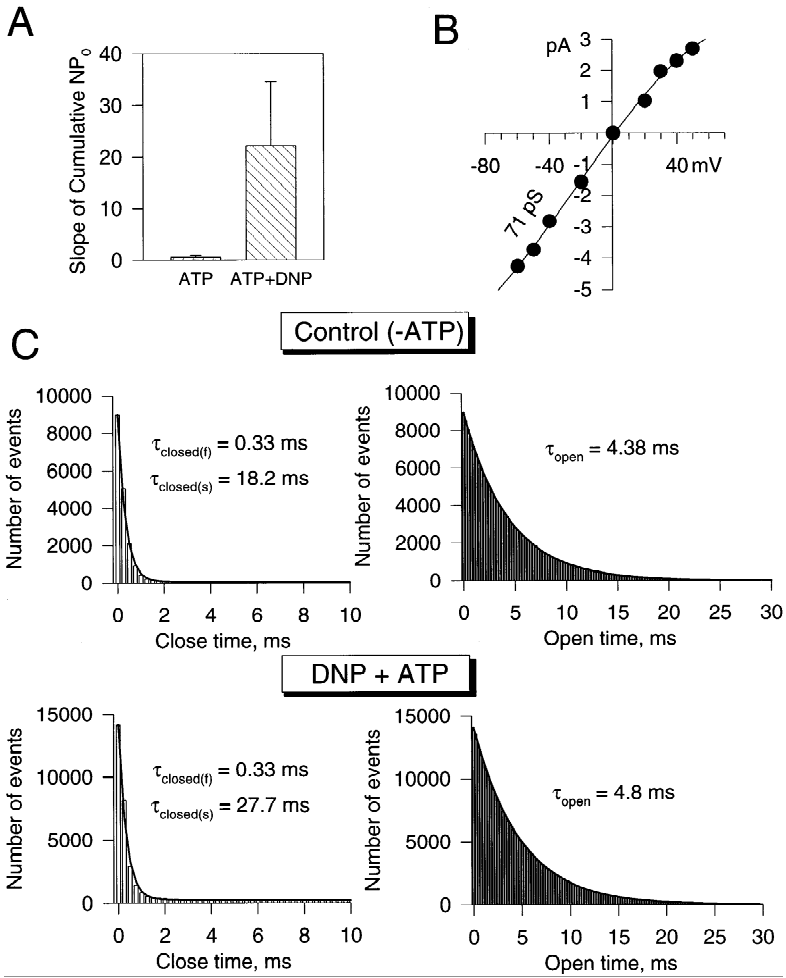

Fig. 2. Properties of DNP-induced K_{ATP} current. (*A*) Summarized data ($n = 4$) of K_{ATP} channel activity (expressed as the slope of cumulative NP_0) in 1 mM ATP alone (left column), and in ATP plus 200 μM DNP (right column). Experimental protocol as in Fig. 1*C*. (*B*) Voltage-current relationship obtained for the K_{ATP} channel in the presence of 200 μM DNP and 2 mM ATP. Within the range of potential −60 to 0 mV, single-channel conductance was 71 pS for the linear part of the voltage-current relationship. At positive potentials the voltage-current relationship deviated slightly from a linear dependence on membrane potential, a specific feature of the moderately inwardly-rectifying K_{ATP} channel. (*C*) Kinetic properties of spontaneous and DNP-induced K_{ATP} channels. Close and open time distributions obtained from the analysis of K_{ATP} channel activity within the same patch. Data are presented with a bin width size of 250 μsec. Close time distribution was fitted by two exponential functions:

$$\Psi(t) = A_1 \exp(^{-t}/_{\tau_1}) + A_2 \exp(^{-t}/\tau_2)$$

where the sum of the relative area under each exponent A_1 and A_2 equals the total number of events, and τ_1 and τ_2 correspond to the time constants of each exponent. Open time distribution was fitted by one exponential function. Results of data fitting are plotted as solid lines.

down of spontaneous K_{ATP} channel activity, DNP alone (200 μM) failed to induce channel opening even after prolonged exposure (>5 min), but after addition of ATP (1 mM), K_{ATP} channel activity, expressed as the slope of cumulative NP_0, increased from 0.06 to 9.1 min^{-1} (Fig. 1*D*; $n = 4$).

Properties of DNP-induced K_{ATP} Channel Openings

The DNP-induced current, under symmetrical K^+ concentrations on the intra- and extracellular side, showed a reversal potential at 0 mV (Fig. 2*B*). Between −60 to 0 mV, single channel conductance was at ~71 pS, and at depolarizing potentials the voltage-current relationship moderately deviated from linear dependence (Fig. 2*B*). At a holding potential of −50 mV, single channel amplitude, estimated by analysis of amplitude distribution, was 3.7 ± 0.3 pA in the absence and 3.5 ± 0.2 pA in the presence of DNP ($n = 3$). Open time histograms (Fig. 2*C*) could be fitted by one exponential function with a time constant (τ_{open}) at 4.4 msec in control (absence of DNP or ATP), and 4.8 msec in DNP (200 μM plus 1 mM ATP). Closed time histograms could be fitted by two exponential functions (Fig. 2*C*). The faster time constant ($\tau_{closed(f)}$) was 0.33 msec both in control and in DNP plus ATP. The slower time constants ($\tau_{closed(s)}$) were 18.2 msec in control, and 27.7 msec in DNP plus ATP, parameters within the range of previously characterized K_{ATP} channels (Terzic, Jahangir & Kurachi, 1995). The existence of the slower component (which relates to interburst time distribution) may relate to the sensitivity of DNP-induced channels toward ATP which increases the time between bursts of K_{ATP} channel opening (Nichols & Lederer, 1991).

After rundown of spontaneous K_{ATP} channel activity, DNP (200 μM) rapidly induced (within ~30 sec) K_{ATP} channel opening in the presence of 2 mM ATP. In the patch depicted in Fig. 3*A*, combined application of DNP and ATP did not affect I_{KI} channel activity. Upon washout of DNP and ATP, K_{ATP} channel activity disappeared (Fig. 3*A*). This suggests that the observed channel activity was not the result of MgATP-induced restoration of channel openings, since increase

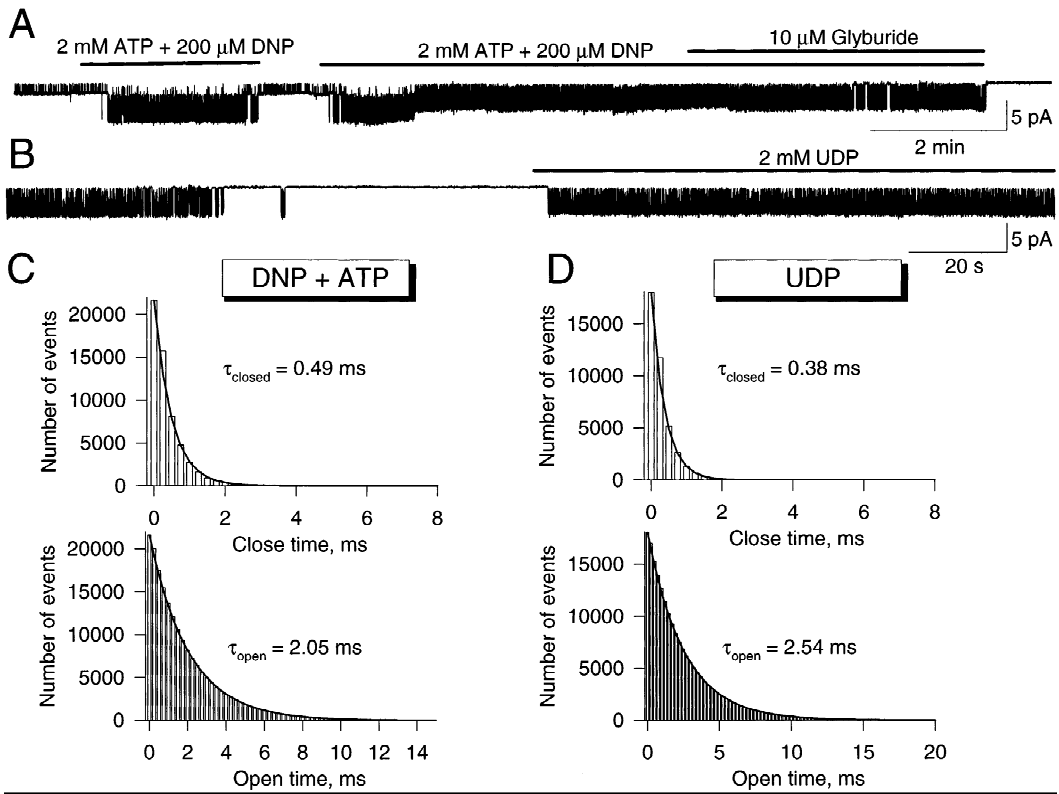

Fig. 3. DNP-induced K_{ATP} channels in the presence of ATP following rundown. (*A*) After the spontaneous K_{ATP} channel activity had ''rundown'', addition of 2 mM ATP with 200 μM DNP reopened K_{ATP} channels. A lower conductance channel activity, characteristic of I_{KI} channels, was present initially and was apparently not affected by DNP, but spontaneously disappeared during the second application of DNP. Glyburide (10 μM) failed to inhibit DNP-induced channel activity. Holding potential was −60 mV. (*B*) Following the rundown of spontaneous K_{ATP} channel activity, UDP induced channel opening. Holding potential was −60 mV. (*C* and *D*) Close and open time distributions were fitted by one exponential function. Results of data fitting are plotted as solid lines.

in K_{ATP} channel activity would have been expected upon removal of ATP (Takano & Noma, 1993; Findlay, 1994; Terzic, Tung & Kurachi, 1994d). Further application of DNP, in the presence of ATP, re-activated K_{ATP} channel openings (Fig. 3*A*) which was observed in 6 out of 9 so-tested patches. This channel activity was not abolished by glyburide (10 μM; Fig. 3*A*), but did possess single channel conductance and kinetic properties of cardiac K_{ATP} channels restored after rundown (Fig. 3*C*).

Nucleoside diphosphates, such as uridine diphosphate (UDP; Fig. 3*B*), can also restore K_{ATP} channel activity after rundown (Tung & Kurachi, 1991; Terzic et al., 1994*a;* 1995). Distributions of open and closed times for DNP- and UDP-induced K_{ATP} channel activity were characterized by an apparent absence of interburst intervals (Fig. 3). Closed time distributions could be fitted by one exponential with time constants (τ_{closed}) 0.49 msec (DNP plus ATP; Fig. 3*C*), and 0.38 msec (UDP; Fig. 3*D*). Open-time histograms could be fitted by one exponential function with time constants (τ_{open}) 2.05 msec (DNP plus ATP; Fig. 3*C*) and 2.54 msec (UDP; Fig. 3*D*).

pH Dependence of the Effect of DNP on Whole-cell K_{ATP} Current

Since the membrane permeability of DNP strongly depends upon pH (Liberman et al., 1969; McLaughlin & Dilger, 1980), the effect of DNP was determined at various extracellular pH values. At pH 8.0, Tyrode solution alone failed to induce significant outward current (Fig. 4*A*, traces 1–2), yet prevented induction of outward current by DNP (trace 3). At pH 7.4, DNP induced outward K^+ current (trace 4), which was abolished upon return to pH 8.0 (Fig. 4*A*). Subsequently, washout of DNP with Tyrode at pH 7.4 (20 min-point Fig. 4*A*), led to transient reactivation of outward current resulting from delayed washout of DNP from the membrane. After restoration of control currents (Fig. 4*A*, trace 5), addition of DNP at pH 6.4 led to rapid development of a large outward current (trace 6), ~5 times faster than the current induced by DNP at pH 7.4 (trace 4). On average, the magnitude of the maximal DNP-induced current was 0.1 ± 0.1 (n = 3), 2.1 ± 0.3 (n = 9), and 2.4 ± 0.5 nA (n = 3) at extracellular pH of 8.0, 7.4 and 6.4, respectively. Average

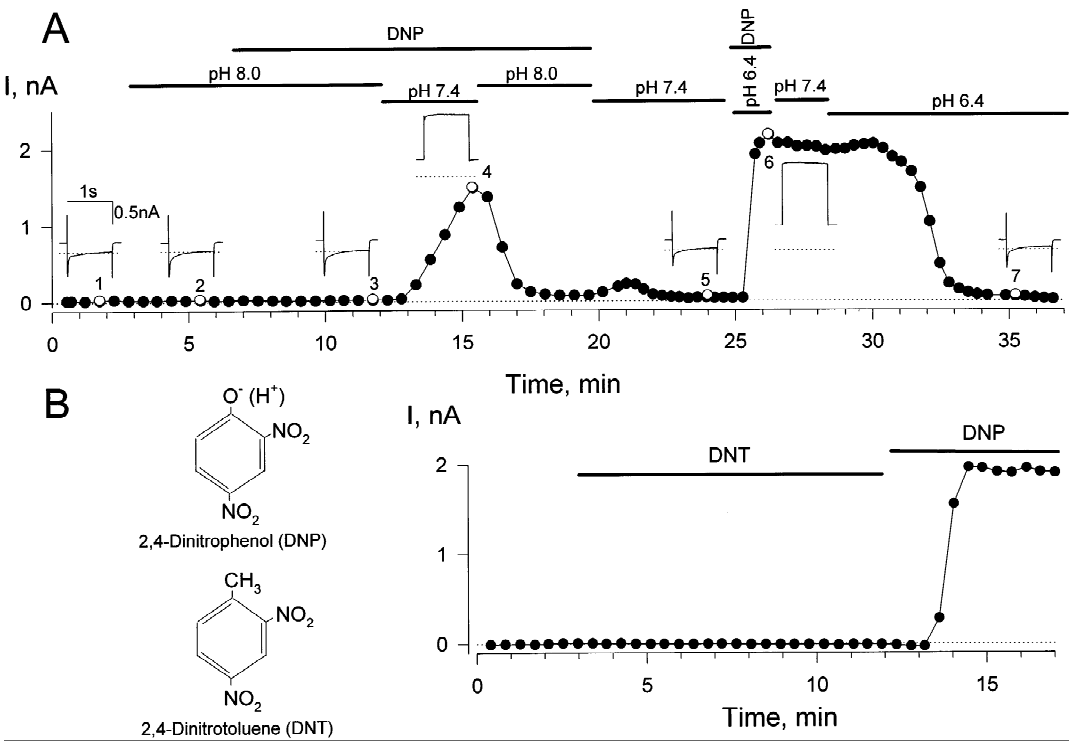

Fig. 4. DNP (200 μM), but not its analogue DNT (200 μM), activates K_{ATP} current in a pH-dependent manner under the whole-cell mode. (*A*) Current values were obtained at the end of a 1,000-msec long depolarizing pulse applied from a holding potential of −50 to 0 mV, and plotted as function of time. Fur current values labeled with an open circle and numbered, original current records are provided. (*B*) Chemical structures of DNP and DNT are provided for comparison. Current values were obtained as in *A.* The value of the extracellular pH was 7.2.

times required for development of DNP-induced current were 585 ± 106 s (at pH 7.4; $n = 9$) and 78 ± 34 sec (at pH 6.4; $n = 3$). Washout of DNP with Tyrode at pH 7.4 (26 min-point) induced a decrease in outward current which was transiently reversed by switching to pH 6.4 prior to restoration of control currents (at 35 min; trace 7; Fig. 4*A*). Thus, the effect of DNP was marginal at an extracellular pH remote from its pK value (~4.1; Markin et al., 1969), but increased as the extracellular pH approached this value in agreement with the pH-dependence of the membrane permeability of DNP.

DNP-analogue Lacking the pH-Titrable Hydroxyl Group Does Not Activate K_{ATP} Current

2,4-Dinitrotoluene (DNT) is a DNP analogue, in which the pH-titrable hydroxyl-moiety is replaced by a methyl group, and thus lacks protonophoric properties (Fig. 4*B*). Application of DNT (200 μM) to whole-cell voltage-clamped cardiomyocytes failed to induce outward current (Fig. 4*B*). In contrast, application of DNP induced large outward current under same conditions (Fig. 4*B*).

Effect of Distinct Protonophores on K_{ATP} Current

Similar to DNP, FCCP (1 μM), a weak acid capable of shuttling protons across membranes (Benz & McLaughlin, 1983), when applied at pH 7.4, induced an outward current of average amplitude 2.2 ± 0.4 nA ($n = 4$; Fig. 5*A*). Alkalinization of the extracellular solution to pH 8.0 led to partial inhibition of this effect, as the magnitude of the outward current decreased to 1.6 ± 0.3 nA ($n = 3$; Fig. 5*A*). Washout of FCCP at pH 7.4, led to transient activation of an outward current related to slower washout of FCCP from the membrane compared to the rate of change of extracellular pH, with recovery of control currents within a 15 to 20-min washout period (Fig. 5*A*). Thus, FCCP shares the property of DNP to activate an outward current in a pH-dependent manner.

In contrast to DNP and FCCP, the antibiotic nigericin (2 μM), which uncouples oxidative phosphorylation by exchanging H^+ for K^+ (Barreto & Lichtenberger, 1992; Bernardi et al., 1992), failed to activate K_{ATP} current at extracellular pH of 6.4 (Fig. 5*B; n* = 4). Yet, in the same patch, DNP did activate K_{ATP} channels (Fig. 5*B*). DNP-induced K_{ATP} channel activity, measured under cell-attached mode, was not modified by changes in

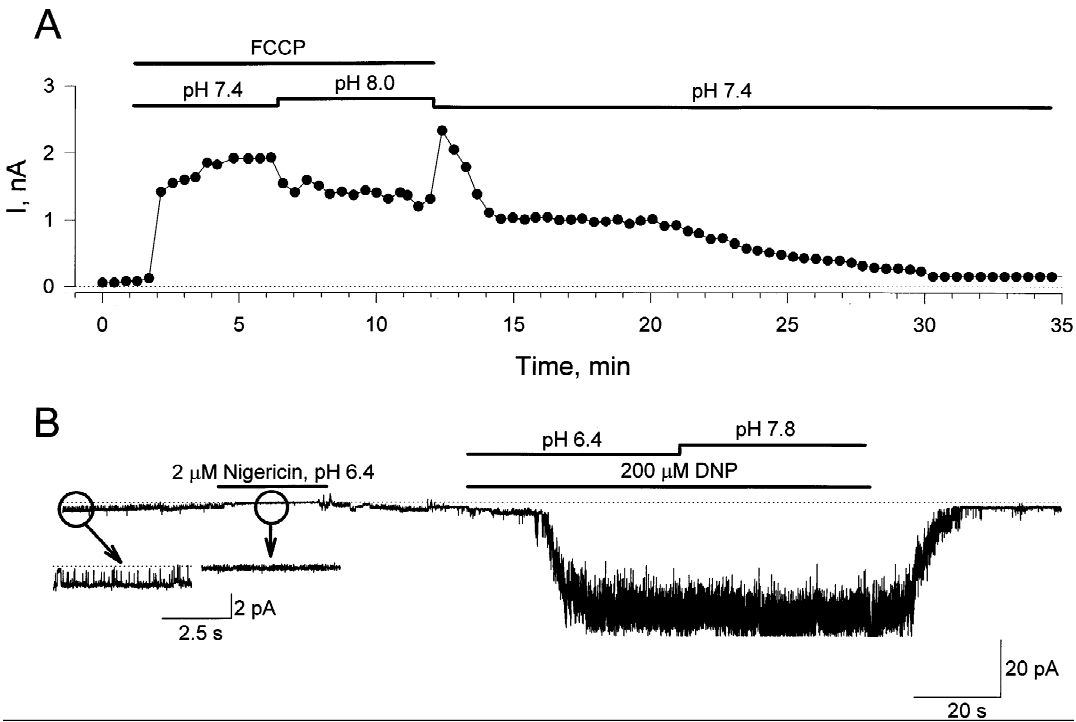

Fig. 5. pH-dependence of K_{ATP} current induced by a weak acid vs. an electroneutral ionophore. (*A*) pH-dependence of the effect of the weak acid FCCP (1 μM) on outward K^+ current under the whole-cell mode. Current values were obtained at the end of a 1,000-msec long depolarizing pulse applied from a holding potential of −50 to 0 mV. (*B*) Effects of the antibiotic nigericin (2 μM) and the weak acid DNP (200 μM), in the cell-attached mode. The cardiomyocyte, bathed in internal solution at pH 7.3, was held at −60 mV. Dotted line represents zero current level. Nigericin, added to the bath solution (pH 6.4), inhibited the inward rectifying channel (I_{KI}), but failed to activate K_{ATP} current. DNP, also added to the bath solution, induced vigorous K_{ATP} channel opening which under the cell-attached mode was not affected by change in the extracellular pH.

extracellular pH from 6.4 to more alkaline values (Fig. 5*B*). Thus, the common properties of DNP and nigericin to uncouple oxidative phosphorylation could not have been responsible for DNP-induced K_{ATP} channel activation under these experimental conditions.

pH Dependence of DNP-induced Single K_{ATP} Channels in Excised Patches

ATP (1 mM) inhibited spontaneous K_{ATP} channel activity both at pH 7.3 ($n = 17$) and 6.4 ($n = 4$; Fig. 6). Application of DNP (200 μM) in the presence of ATP at pH 7.8 ($n = 3$) from the internal side of the patch did not evoke K_{ATP} channel activity. In the presence of ATP at pH 6.4, addition of DNP, to the internal side of the patch, led to transient activation (~40 sec) of K_{ATP} channel opening (Fig. 6). Alkalinization to pH 7.8 ($n = 2$), in the presence of DNP and ATP, induced sustained openings of K_{ATP} channels (slope of cumulative NP_0: 1.83 min^{-1}; Fig. 6). Acidification to pH7.3 diminished the ability of DNP to induce channel openings in the presence of ATP (slope of cumulative NP_0: 0.97 min^{-1}; Fig. 6). Thus, the pH dependence of DNP-induced K_{ATP} channel openings was reversed relative to whole-cell experiments (Fig. 4*A*) which is not consistent with simple diffusion of a molecule of DNP across the sarcolemma.

Molecular Forms of DNP

Indeed, transfer of DNP across a membrane does not occur by simple diffusion (Markin et al., 1969). Rather, DNP transfers as a net result of bidirectional movement of neutral and charged forms of this weak acid (McLaughlin, 1972; Benz & McLaughlin, 1983). Within the thin layer of membrane interface, a molecule of DNP (T^-) and hydrogen form a monomer ($M = H^+ + T^-$), which, in turn, can form a dimer ($M_2^- = M + T^-$; Poonia, 1975). Due to the strong hydrophobic adsorption of T^- and M (McLaughlin, 1972; Dilger & McLaughlin, 1979), M_2^- is formed at the membrane interface where concentrations of T^- and M are much higher than at the aqueous or membrane faces. At equilibrium, dissociation constants of the monomer (ζ_M) and dimer (ζ_D) complexes are defined as:

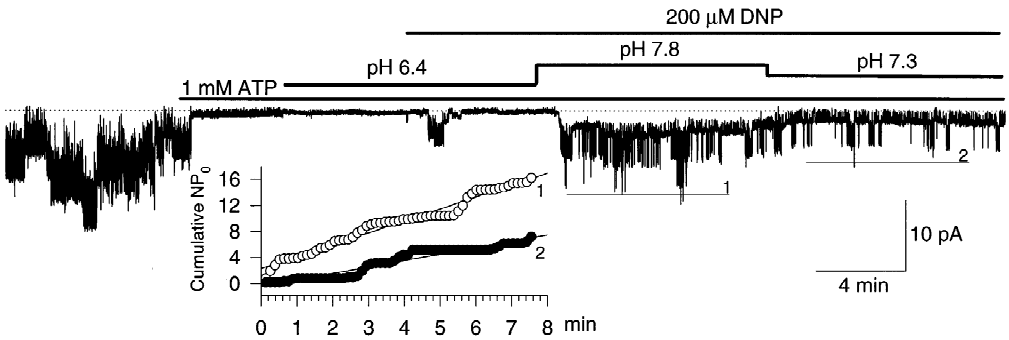

Fig. 6. pH-dependence of the effect of DNP on K_{ATP} channels in cell-free patches. Following membrane excision, spontaneous K_{ATP} channel activity was blocked by 1 mM ATP which remained suppressed after switching the pH of the internal solution from 7.3 to 6.4. DNP, at pH 6.4, induced transient opening of K_{ATP} channels, which became sustained at pH 7.8, and partially decreased at pH 7.3. *Inset:* DNP-induced K_{ATP} channel activity obtained at pH 7.8 (open circles) and 7.3 (filled circles) was quantified by calculating the cumulative NP_0 values corresponding to segments, labeled 1 and 2, of original trace (denoted by horizontal bars beneath the original trace). Results of linear regression (estimated linear correlation coefficients 0.97 and 0.92 for segments 1 and 2, respectively) are presented as solid lines.

$$\zeta_M = \frac{[H^+][T^-]}{[M]}, \quad \zeta_D = \frac{[M][T^-]}{[M_2^-]} \tag{1}$$

Due to its larger radius, but identical charge when compared to T^-, M_2^- has a higher membrane permeability as predicted by the Born equation:

$$W = \frac{e^2}{8\pi\varepsilon_o r}\left\{\frac{1}{\varepsilon_2} - \frac{1}{\varepsilon_1}\right\}$$

which relates the electrostatic energy (W) required to move a spherical ion of charge e and radius r from the aqueous phase (dielectric constant: ε_1) into the membrane (dielectric constant: ε_2).

When the total concentration (T^{Tot}) of DNP is lower than the dissociation constant ζ_D, as is the case under our experimental conditions, the concentration of M_2^- is much lower than M or T^-, and T^{Tot} is approximately equal to T^- plus M. The concentration of M_2^-, can be calculated from Eq. (1) as:

$$[M_2^-] = \frac{\zeta_M}{\zeta_D}[T^{Tot}]^2 \frac{[H^+]}{(\zeta_M + [H^+])^2} \tag{2}$$

Applying Eqs. (1) and (2) to our experimental conditions indicated that the pH-dependence of the concentrations of T^- and M was monotonic in contrast to that of M_2^- which was bell-shaped with a maximum at pH = pK (Fig. 7; Liberman & Topaly, 1968; Lea & Croghan, 1969). The concentration of M_2^- was significantly lower than that of M at all pH values (Fig. 7), including the pH range used in this study (Fig. 7 inset). The estimated concentration of M was >1 μM at pH 6.0 (pH > pK), but decreased to ~0 μM at pH ≥ 8.0 (pH >> pK). If the permeant forms of DNP (M and M_2^-) are responsible for activation of K_{ATP} channels then concentrations of several μM of M and/or M_2^- are apparently sufficient for the action of DNP.

Discussion

This study demonstrates that DNP can open cardiac K_{ATP} channels in the presence of millimolar concentrations of intracellular ATP, even under cell-free conditions. Thus, DNP could activate myocardial K_{ATP} channels through a mechanism separate from inhibition of mitochondrial oxidative phosphorylation.

Under whole-cell configuration, the effect of DNP was reversible and reproducible without cell contracture (*see also* Findlay, 1993), and occurred in a timeframe within which major changes in high energy phosphates have not been reported (Weiss & Hiltbrand, 1985). DNP-induced opening of K_{ATP} channels was associated with suppression of Na^+ and Ca^{2+} currents, which are known to be sensitive to metabolic stress. Yet, such changes also occur with K^+ channel openers without metabolic inhibition (Terzic, Jahangir & Kurachi, 1994*B*). Furthermore, in the continuous presence of DNP blockade of K_{ATP} current does not have major effect on Na^+ and Ca^{2+} currents (Mejia-Alvarez & Marban, 1992; Wu et al., 1992). Thus, depletion of intracellular ATP may not be a prerequisite for DNP-induced K_{ATP} channel opening. As localized fluctuations in ATP concentration cannot be ruled out, direct evidence for dissociation between ATP depletion and K_{ATP} channel opening was obtained in cell-free patches.

The finding that the pH-titrable hydroxyl group appeared essential for DNPs action, since replacement by a methyl-moiety led to loss of K_{ATP} current activation, is consistent with the protonophoric features of DNP (Liberman & Topaly, 1968; Lea & Croghan, 1969; McLaughlin, 1972). Moreover, the pH-dependence in

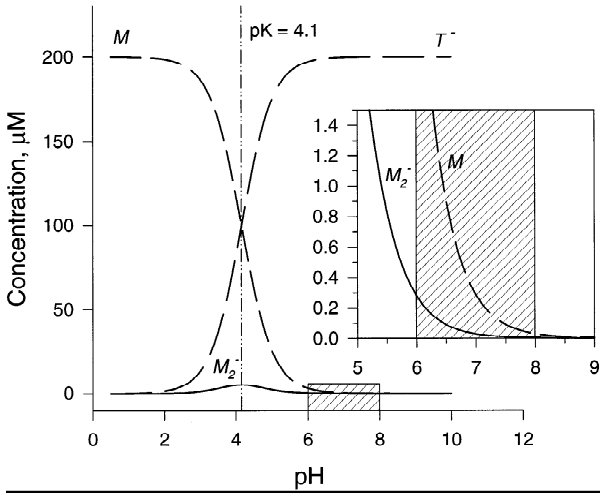

Fig. 7. Concentration of DNP forms as a function of pH. Concentrations of DNP forms (M, M_2^-, T^-) were calculated, in terms of pH, based on Eqs. (1) and (2) as presented in the Results:

$$[M] = [T^{Tot}]\frac{1}{1+10^{-pH}/\zeta_M},$$

$$[T^-] = [T^{Tot}]\frac{10^{-pH}/\zeta_M}{1+10^{-pH}/\zeta_M},$$

$$[M^-_2] = \frac{\zeta_M}{\zeta_D}[T^{Tot}]^2\frac{10^{-pH}}{(\zeta_M+10^{-pH})^2}$$

where the total concentration of DNP: $[T^{Tot}] = 2{\cdot}10^{-4}$ M; the dissociation constant of the M form: $\zeta_{MESC} = 7{\cdot}10^{-5}$M; and the dissociation constant of the $M2$ form: $\zeta_D = 2{\cdot}10^{-3}$ M. Using these parameters, maximal concentration of the M_2^- form is at pH = pK. (4.1). The concentration of the M_2^- form was calculated assuming that the total concentration of DNP is primarily determined by T^- and M. The range of pH values used in the study is shaded, and magnified as an *inset.* At pH 6.4, concentrations of the M_2^- and M forms were 0.11 and 1.13 μM, respectively, whereas at pH 7.4 they were 0.011 and 0.11 μM, at pH 7.8 they were 0.005 and 0.05 μM, and at pH 8.0 they were 0.003 and 0.03 μM, respectively.

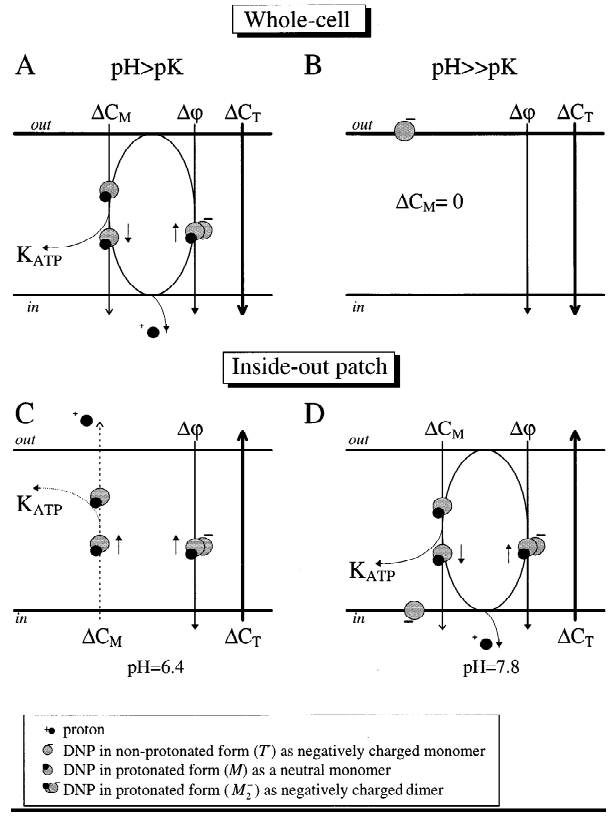

Fig. 8. Transfer of DNP forms across the membrane and channel activation. Three forms of DNP, the permeant, M and M_2^-, and the poorly permeant, T^-, are represented by symbols. ΔC_T, ΔC_M and $\Delta\varphi$ denote concentration gradients for T^- and M, and the applied electrical gradient, respectively. *Out* and *in* relate to extra- and intracellular sides of the sarcolemma. (*A*) At extracellular pH > pK (e.g., at pH 6.4 – 7.4), M transfers down the concentration gradient from *out* to *in*, whereas M_2^- transfers from *in* to *out* driven by the electrical gradient. Bidirectional transfer of two M for each M_2^- leads to net transport of one proton from *out* to *in*, and K_{ATP} channel activation. (*B*) At extracellular pH >> pK (e.g., at pH 8.0), the lack of free protons at the extracellular side of the membrane increases the concentration of T^-. (*C*) At internal pH 6.4, the concentration gradient drives M across the membrane. As M and M_2^- transfer in the same direction, ΔC_M dissipates resulting in transient activation of K_{ATP} channels (dotted lines). Upon equilibration, M cannot cross any longer the membrane, whereas transfer of M_2^- from *in* to *out*, continues to maintain this equilibrium. (*D*) Alkalinization to 7.8 decreases the concentration of M at the inner side of the membrane (in favor of T^-), in turn creating a concentration gradient for M from *out* to *in*, promoting transfer of M and K_{ATP} channel activation.

the efficacy of DNP-induced K_{ATP} channel activation is in accord with the pH-dependence of DNP-induced membrane conductance which relates to distribution of permeant and nonpermeant forms of this weak acid across a membrane (Liberman et al., 1969).

At acidic extracellular pH (6.4), the permeant form M is present in micromolar concentration (Fig. 7), and can cross the membrane down its concentration gradient (ΔC_M, Fig. 8*A*). This gradient is maintained by transfer in the opposite direction (up the electrical gradient) of M_2^-, the other permeant form of DNP (Fig. 8*A*). At alkaline pH (8.0), the concentration of M is ~0 μM (Fig. 7), and net transfer of DNP across the membrane stops (Fig. 8*B*). Thus, differences in concentrations of M at acidic vs. alkaline pH, and thereby differences in DNP transfer across the membrane, may underlie changes in the efficacy of DNP to activate K_{ATP} current at various extracellular pH values (*see* Fig. 4*A*). Even after washout of DNP, switching extracellular pH affected K_{ATP} current size (*see* Fig. 4A, 20th and 26th min), consistent with a pH-dependent membrane redistribution of DNP species. FCCP was also less effective at activating K_{ATP} current at more alkaline extracellular pH. But, the efficacy of FCCP was only partially lost at pH 8.0 as at this pH, the concentrations of permeant species of FCCP are higher than those of DNP due to differences in pK values be-

tween FCCP (6.0 to 6.4; Benz & McLaughlin, 1983) and DNP (4.1).

In cell-attached patches at pH 6.4, initiation of K_{ATP} channel activity by DNP (Fig. 5*B*) could also be attributed to transfer of permeant species of DNP across the membrane, and distribution in the vicinity of the channels under the pipette. Extracellular alkalinization reduces the concentration of permeant species of DNP around the whole cell membrane (Fig. 8*B*), but not beneath the pipette. Accordingly, in the cell-attached mode of recording, the efficacy of DNP to induce K_{ATP} channel openings was not reduced following alkalinization of the extracellular milieu (Fig. 5*B*). This indicates that the permeability of the whole membrane towards DNP is not essential for channel activation, as expected if DNP was acting through intracellular metabolic poisoning. Rather, local distribution of DNP forms in the vicinity of recorded channels appeared essential for DNP action, i.e., the effect of DNP was membrane-delimited.

Under cell-free conditions, the pH-dependence of the DNP effect was reversed in direction from that observed under whole-cell conditions. At internal pH 6.4, a micromolar concentration of *M* is present at the inner side, and at first crosses the membrane down its concentration gradient. However, this gradient cannot be sustained due to absence M_2^- transfer in the opposite direction (Fig. 8*C*). Transfer of M_2^- (up the electrical gradient), in the same direction as *M*, leads to dissipation of the concentration gradient for *M* (ΔC_M), and at steady-state in cell-free conditions this is associated with loss of K_{ATP} channel activation at acidic intracellular pH (Fig. 6). Intracellular alkalinization, by reducing the concentration of *M* at the inner side of the sarcolemma, induces ΔC_M leading to transfer of *M* from outside to inside (Fig. 8*D*). This is consistent with K_{ATP} channel activation observed under this condition (pH 7.8; Fig. 6). This suggests a critical role for transfer of *M* in the activation of K_{ATP} channels.

Movement of the molecular forms of DNP across the membrane could have several consequences. Transfer of protons by DNP may increase proton concentration at the inner side of the membrane so activating K_{ATP} channels (*see* Davies, Standen & Stanfield, 1992; Fan & Makielski, 1993; Koyano et al., 1993; Vivaudou & Forestier, 1995; Allard, Lazdunski & Rougier, 1995). However, the effect of DNP is probably not solely related to changes in intracellular pH as in cell-free patches alkalinization of the inner side of the membrane increased, whereas acidification decreased DNPs efficacy in opening K_{ATP} channels. These findings may suggest a role for the protonophore itself in channel activation perhaps through protonation of critical transmembrane amino acid residues within the K_{ATP} channel complex, a view supported by the finding that substitution of the proton donating group of DNP led to loss of K_{ATP} channel activation property.

Furthermore, DNP could have interacted with K_{ATP} channels as a ligand, and the pH-dependence of its action related to the membrane distribution of the active form critical to activate K_{ATP} channels. For instance, T^- could be the active form since permeation of DNP across the sarcolemma would lead to increase in its concentration at the inner side of the membrane. However, this is unlikely since a different efficacy of DNP to activate K_{ATP} channels was observed (Fig. 4 and 6) in a pH-range within which the concentration of T^- essentially remains constant (Fig. 7), suggesting that the permeant are the active form(s). An active form of DNP may share properties with known K^+ channel opening drugs which induce K_{ATP} channel activation through not only pH^- (Edwards & Weston, 1993; Forestier, Pierrard & Vivaudou, 1996; Jahangir, Terzic & Kurachi, 1994; Terzic et al., 1995), but also ATP-dependent mechanism(s) (Terzic et al., 1994bc). Moreover, the kinetic properties of DNP- and UDP-induced openings of K_{ATP} channel were similar (Tung & Kurachi, 1991; Findlay, 1994; Terzic et al., 1994*a*, 1995). As UDP-induced channel openings are blocked by ATP and glyburide (Tung & Kurachi, 1991; Terzic et al., 1994*a*), whereas DNP-induced openings were not, it is conceivable that DNP may possess unique K_{ATP} channel opening properties.

Finally, DNP alters the electrostatic potential at the surface of membranes (McLaughlin & Dilger, 1980) induced at the hydrodynamic plane of shear (Eisenberg et al., 1979). This could be of importance for its action since surface charges may gate K_{ATP} channels (Deutsch, Matsuoka & Weiss, 1994; Vivaudou & Forestier, 1995).

Although we cannot determine a precise mechanism of DNPs interaction with the K_{ATP} channel, the loss of channel sensitivity to ATP, may suggest that the active form(s) of DNP act(s) through a pathway leading to functional uncoupling of the ATP-channel gating from the pore region (Aguilar-Bryan et al., 1995; Inagaki et al., 1995; Nichols et al., 1996). Our data suggest that the pore region of the K_{ATP} channel is an unlikely target for DNPs action as DNP did not alter channel conductance nor the behavior in terms of intraburst kinetics. On the other hand, nucleotide-binding sites are separate from the pore region of the channel, and localized on the regulatory subunit of the channel (Inagaki et al., 1996), suggesting that loss of ATP-sensitivity may be associated with disturbances in the regulatory gating of K_{ATP} channels. Endogenous counterparts of DNP could include intracellular protonophores, such as unsaturated fatty acids (Wang, Richardson & Thayer, 1995) which reduce the duration of action potentials (Kang, Xiao & Leaf, 1995), and weak acids, such as gluconate and pyruvate, which enhance K_{ATP} channel activity in the presence of ATP (McKillen et al., 1994).

In conclusion, DNP can induce K_{ATP} channel opening in the presence of millimolar concentrations of ATP. This further supports previous findings that depletion of intracellular ATP is not a prerequisite for K_{ATP} channel opening in the myocardium (Weiss & Venketash, 1993; Decking et al., 1995; Terzic et al., 1995). Traditionally, DNP has been used to activate K_{ATP} channels and simulate conditions of metabolic compromise. The present results argue for reconsideration of such an approach since DNP opened K_{ATP} channels even under cell-free conditions.

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