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Negative inotropic effect induced by diethylamiloride (DEA) in rabbit myocardium

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Abstract—The effects of the Na^+/H^+ exchange blocking drug diethylamiloride (DEA) on mechanical function have been studied in the rabbit isolated, arterially perfused interventricular septum. At concentrations of 10^{-6}–10^{-5} M, DEA induced a significant, dose-dependent, negative inotropic effect (a 54% decrease from control values at the highest concentration), which was slow to develop. After a 45 min washout, recovery was almost complete (95 ± 3·4%). At concentrations $> 5 \times 10^{-5}$ M, DEA induced a rapid and marked decrease in developed tension, associated with a progressive decrease in excitability and incomplete recovery. Resting tension was not significantly modified at any of the concentrations tested. At $> 10^{-6}$ M DEA enhanced significantly the transient negative inotropic effect of the brief intracellular acidosis induced by removal of NH_4Cl perfusion, both by decreasing the minimal value of developed tension and by increasing the time required to produce this effect. These effects suggest that the dose-dependent DEA negative inotropic effect could be mediated by a progressive intracellular acidosis produced by inhibition of the Na^+/H^+ exchange system.

Diethylamiloride (DEA) is one of the 5-amino-substituted amilorides which exhibit high potency in blocking Na^+/H^+ exchange (Vigne et al 1984). The use of Na^+- and pH-sensitive microelectrodes and $^{22}Na^+$ flux studies has identified this exchange system in sheep Purkinje fibres (Deitmer & Ellis 1980) and dog cardiac sarcolemmal vesicles (Pierce & Philipson 1985). It has been further characterized by the use of this type of inhibitor in cardiac cells in cultures from rat and chick hearts (Vigne et al 1984).

The purpose of this study was to analyse the effects of Na^+/H^+ exchange inhibition by DEA on the mechanical parameters of the interventricular septum of the rabbit myocardium to evaluate the cotransport inhibition.

Material and methods

Experiments were carried out using the isolated arterially perfused interventricular septum of the rabbit (Langer & Brady 1968). Adult New Zealand White rabbits, 2–2·5 kg were heparinized (2000 units) and given an intravenous overdose of pentobarbitone (120 mg). The thorax was opened and heart removed. Within 4 min, the septal artery was cannulated and the interventricular septum immediately perfused at a constant flow (2 mL min^{-1} g^{-1} wet weight) with a roller pump (Gilson, Minipuls HP4). The perfused area of the septum was dissected free and mounted in a Perspex chamber with its base fixed by forceps and its apex attached to a transducer (Grass FT03). The tension was recorded continuously in a model 79D Grass polygraph. Septa were electrically stimulated with pulses of 15 V and duration 5 ms at a rate of 1 Hz (Grass, SD9). Only septa with 10 g or more developed tension and 10 g or less resting tension at 28 C and 0·5 Hz were used in this study. The perfusate was a modified Tyrode solution of the following composition (mM): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1·8, NaH_2PO_4 0·43, D-glucose 5·5 and HEPES 3 (pH 7·4). The solution was bubbled with O_2. The septal temperature was monitored with a thermistor needle inserted directly into the muscle, and maintained at 32 ± 0·3°C by warming the Tyrode solution with a thermostatically controlled heating coil and by heating the chamber with a water-filled radiator through which nitrogen gas was bubbled.

In all experiments, an equilibration period of 45 min was allowed and, to avoid residual effects, only one drug concentration experiment was performed with each septum. After stabilization DEA (10^{-7} 5×10^{-5} M) was added to the perfusate. Once a steady-state response had been obtained, perfusion was changed to a non-drug containing solution. To evaluate the inhibition of Na^+/H^+ exchange activity by DEA an intracellular acidosis was induced by stopping perfusion with NH_4Cl, using the ammonia rebound technique (see Roos & Boron 1981). In brief, NH_4Cl (10 mM) was perfused over 5 min. Removal of NH_4Cl from the perfusate induced a transient acidosis which was evident by a brief negative inotropic effect. Once this negative inotropic effect was over, DEA was perfused in an NH_4Cl containing solution (5 min) and a second acid load was produced in the presence of the drug. The parameters evaluated in this protocol were the minimal value of developed tension during acidosis and its timing, the corresponding results on contractility being expressed as percentage of developed tension obtained at the onset of NH_4Cl perfusion.

The diethyl analogue of amiloride, 3-amino-6-chloro-5-(*N*-diethylamine)-2-pyrazinoylguanidine, was prepared according to previously reported patent procedures (Merck Co. Inc. 1965, 1968).

Results were expressed as the mean ± s.e.m. Differences betweeen groups of experiments were analysed by Student's *t*-test.

Results

The effects of DEA on resting and developed tension of rabbit myocardium septa are shown in Fig. 1. At 10^{-6} 10^{-5} M, DEA induced a significant, dose-dependent, negative inotropic effect (13·1 ± 1·84 g), reaching a 54% decrease at the highest concentration. This effect developed slowly, with the maximal effect occurring 30 min after perfusion with the drug. At these concentrations, after a 45 min washout period, recovery was almost complete (95 ± 3·4%). At $> 5 \times 10^{-5}$ M, DEA induced a rapid and marked decrease in developed tension with the maximal effect observed within 3–4 min of addition of the drug to the perfusate. This effect was associated with a progressive decrease in excitability. After reperfusion with a drug-free solution, septa recovered only partially to < 47 per cent of pre-drug developed tension.

Resting tension was not signficantly modified from control values (8·6 ± 0·6 g) at any of the concentrations tested.

Fig. 2 shows a typical recording of the effects of NH_4Cl in an interventricular septum of rabbit myocardium. NH_4Cl perfusion by itself induces a transient increase in developed tension (Fig. 2a), shorter in duration than the time of drug perfusion. This effect has been described by Bountra & Vaughan-Jones (1989) in mammalian myocardium and it has been related to the transient

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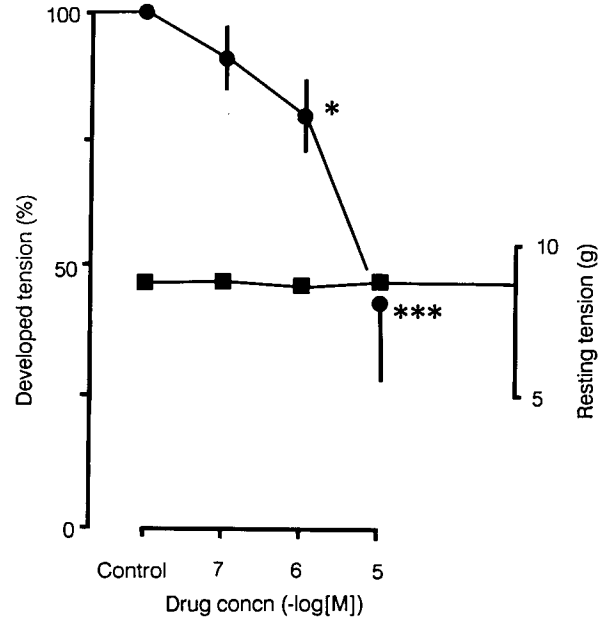

Fig. 1. Effects of 30 min perfusion of diethylamiloride on: (●) developed tension and (■) resting tension in the isolated, arterially perfused interventricular septum of the rabbit myocardium. Control values: 13·1 ± 1·84 g and 8·6 ± 0·6 g, respectively. Each point represents the mean of at least 4 experiments; vertical lines show the s.e.m. * $P < 0.05$, *** $P < 0.001$.

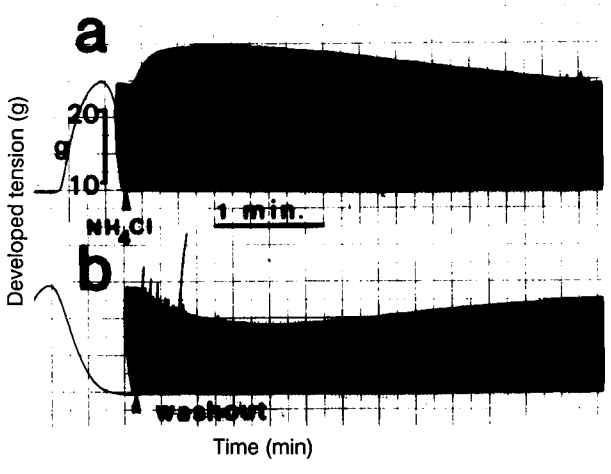

FIG. 2. Typical recording of the effects of NH_4Cl perfusions (a) and of the corresponding acid load induced by NH_4Cl perfusion removal (b) on developed tension obtained in an isolated interventricular septum.

increase of pH observed during pefusion with this drug. Removal of NH_4Cl perfusion induced a 48% decrease in the developed tension compared with control values (11·8 ± 1·94 vs 6·1 ± 1·22 g; n = 12, $P < 0.001$), with the maximal effect occurring 1·4 min after removal of the drug (Fig. 2b, Table 1). In the absence of any other treatment, a second acid load by NH_4Cl removal induced a similar decrease in developed tension (45·4 ± 6·2% vs 43·8 ± 7·2%; n = 4, $P > 0.05$).

The effects of 10^{-7}–10^{-5} M DEA on the transient negative inotropic effect induced by removal of NH_4Cl perfusion are shown in Table 1. DEA $> 10^{-6}$ M enhanced significantly the negative inotropic effect induced by removal of NH_4Cl perfusion, both by decreasing the minimal value of developed tension and by increasing the time required to reach this effect. At the drug perfusion times that this effect was obtained (8·2 and 11·2 min for 10^{-6} M and 10^{-5} M DEA, respectively) the contribution of DEA to the negative effect is significantly greater than that corresponding to the non-acid load experiments already described.

Discussion

The data presented here are consistent with the hypothesis that the dose-dependent DEA negative inotropic effect could be mediated by a progressive intracellular acidosis produced by the inhibition of the main transport mechanism (Na^+/H^+ exchange system) that removes acid from the cell (Roos & Boron 1981). There is good agreement between the range of concentrations that induces the negative inotropic effect and the range which potentiates the transient negative inotropic effect induced by the transient intracellular acidosis due to removal of NH_4Cl perfusion. Such an enhancement induced by amiloride of intracellular acidosis and of the fall in tonic tension has been already shown by the use of pH-selective microelectrodes in sheep cardiac Purkinje fibres (Kaila & Vaughan-Jones 1987). Moreover, the long time required for the maximal decrease in inotropy induced by DEA suggests that the hypothetical effect on pH_i requires previous overpassing of the relatively high buffering capacity of myocardial muscle (Ellis & Thomas 1976). Intracellular acidosis induces a fall in myocardial contractility by both a decrease in the Ca^{2+} sensitivity of the contractile proteins and by depression of the Ca^{2+} loading and the rate of Ca^{2+} accumulation of the sarcoplasmic reticulum as well as Ca^{2+}-induced release of Ca^{2+} (Fabiato & Fabiato 1978; Blanchard & Solaro 1984).

The acidosis-induced negative inotropic effect is independent of any decrease in the slow inward Ca^{2+} current (Poole-Wilson & Langer, 1975). Those authors found a prolongation of the action potential duration (APD) while tension fell by more than 50% in interventricular septal preparations in the case of intracellular acidosis induced by increasing CO_2. In electrophysiological experiments (data not shown) we have been unable to demonstrate a shortening effect of 10^{-5} M DEA, a concentration that induces more than a 50% decline in contractility, on APD. This lack of such an effect, as well as the long time required to reach the maximum effect on inotropy (30 min), makes it unlikely that a receptor-mediated mechanism (i.e. interaction with muscarinic receptors) is involved.

N-5-Disubstituted amiloride derivatives are devoid of any direct activity on Na^+/Ca^{2+} exchange (Frelin et al 1984). Nevertheless, in cultures of chick and rat cardiac cells exposed to high concentrations of ouabain the Na^+/H^+ exchange system is a major pathway for Na^+ uptake and the blockade of this cotransporter by 5-amino substituted amiloride derivatives

Table 1. Effects of diethylamiloride (DEA) on the transient negative inotropic effect induced by removal of perfusion with NH_4Cl (10 mM, 5 min) in the interventricular septum of rabbit myocardium. DT = minimal value of developed tension during the transient negative inotropic effect induced by the acid load. † Time required to reach this value. Results are expressed as mean ± s.e.m. of (n) experiments. * $P < 0.001$ when compared with values from NH_4Cl experiments.

	(n)	DT (g)	(% over control)	Time† (min)
Control	(12)	11·8 ± 1·94	—	—
NH_4Cl	(12)	6·10 ± 1·22	(52·0)	1·4 ± 0·17
NH_4Cl + DEA 10^{-7}M	(2)	5·88 ± 1·12	(49·8)	1·5 ± 0·20
NH_4Cl + DEA 10^{-6}M	(6)	4·74 ± 1·47*	(40·2)	3·2 ± 0·46*
NH_4Cl + DEA 10^{-5}M	(4)	1·78 ± 0·25*	(15·1)	6·2 ± 1·34*

decreased $^{45}Ca^{2+}$ entry via Na^+/Ca^{2+} exchange (Frelin et al 1984). The contribution to the negative inotropy of DEA in a working preparation without digitalis treatment, mediated by an indirect inhibition of the Na^+/Ca^{2+} exchange system deserves further study.

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Improved method for morphine determination in biological fluids and tissues: rapid, sensitive and selective

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Abstract—Morphine was assayed using a simple two step solvent extraction—acid back extraction sample preparation method, coupled with normal phase high-performance liquid chromatography (HPLC) and dual electrode coulometric detection. HPLC is performed with a 1·0 M Tris–methanol (5:95) mobile phase with subtle pH adjustments to separate morphine and internal standard from any interfering compounds. The use of normal phase HPLC (silica column) substantially reduces problems from interfering lipophilic substances sometimes encountered with reverse phase HPLC following solvent extraction and which would otherwise require more time-consuming sample preparation. Dual electrode detection further improves the selectivity for morphine and gives excellent sensitivity (0·5 ng mL^{-1}), reproducibility and stability for automated sample injection. This method has proven suitable for pharmacokinetic studies of morphine.

The conclusions of many studies of morphine concentration in biological samples have been unsound because of the unintentional co-measurement of morphine conjugated metabolites. Thus a critical issue in the design of new analyses is selectivity. Comprehensive investigations of the pharmacokinetics and the pharmacodynamics of morphine are only possible if the selectivity towards morphine is assured.

Numerous methods have been published for quantitating morphine in biological fluids and tissues. These have been based upon gas-liquid chromatography following derivatization (Dahlström et al 1977; Felby 1979; Jones et al 1984), reverse phase HPLC following solid phase or solvent extraction with electrochemical (Bolander et al 1983; Moore et al 1984; Derendorf & Kaltenbach 1986; Svensson 1986), UV-VIS (Svensson et al 1982; Säwe et al 1985) or fluorescence detection (Nelson et al 1982; Tagliaro et al 1985), or radioimmunoassay (RIA) methodology (Morris 1975; Hahn et al 1979; Stanski et al 1982; Edwards et al 1986). Most of these methods require several time-consuming sample preparation steps before quantitation. These normally include at least two extractions followed by solvent evaporation and reconstitution to clean up and concentrate the sample extract or, in the case of derivatization, to remove excess reagent and isolate the derivative for measurement. Detection by UV (Svensson et al 1982; Säwe et al 1985) is preferred when both the 3- and 6-glucuronide metabolites of morphine are required to be determined, but many recent methods for morphine alone have utilized amperometric detection with good sensitivity. The use of a dual electrode coulometric detector has, in our experience, given much better long term baseline stability at high sensitivities than the glassy carbon sandwich electrochemical cells. This has allowed overnight automated sample runs, increasing laboratory output without compromising sensitivity or reproducibility.

The initial application of this method was to the determination of the regional clearances and tissue solubility of morphine at steady-state in sheep (Sloan et al 1990). The method is now used routinely for monitoring morphine disposition in a pain management setting.

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