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The Effect of Diphenylamine-2-carboxylate on Cl^- Channel Conductance and on Excitability Characteristics of Rat Skeletal Muscle

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Abstract—The effect of diphenylamine-2-carboxylate (DPC), a blocker of the Cl^- conductive pathway in Cl^- transporting epithelia, has been evaluated in-vitro on the electrophysiological variables of rat extensor digitorum longus muscle fibres. DPC (5–240 μM) caused a dose-related increase of membrane resistance which was attributed entirely to a fall in Cl^- channel conductance (IC50, 120 μM), since potassium conductance was not affected by the treatment. DPC also modified fibre excitability. A significant dose-dependent increase was observed in the latency of the action potential and in the excitability of the membrane. DPC was less potent on striated fibres than anthracene-9-carboxylic acid, another specific blocker of Cl^- channel conductance. Moreover DPC was less potent on skeletal muscle than on Cl^- transporting epithelia. Morphological differences in the Cl^- channels or of the drug binding sites may account for the differences between tissues.

A large chloride conductance (GCl) functions to stabilize mammalian skeletal muscle fibre membranes. Striated fibres with a reduced GCl become hyperexcitable, and produce trains of action potentials such as occur in some muscle diseases including myotonia (Adrian & Bryant 1974; Rüdel & Lehmann-Horn 1985). Nevertheless, little is known about the channel mechanism, at the molecular level, that would explain the large resting GCl (Bretag 1987). Blatz & Magleby (1986), using the patch-clamp recording technique on rat myotubes, described three Cl^- selective channels, one of which had fast kinetics. Although having several properties consistent with those of the resting GCl, the unusual voltage-dependence of this channel, makes it unlikely that it contributes to the large membrane GCl. Moreover, the abundance of Cl^- channels in the rat myotubes is not mirrored by a large macroscopic GCl in those cells (Bretag 1987). Most of the information about this anionic channel has been derived from pharmacological studies. Some drugs, such as anthracene-9-carboxylic acid (9AC), are able to decrease membrane GCl in mammalian striated fibres by binding to an intramembrane site and thereby causing occlusion of Cl^- channels (Bryant & Morales-Aguilera 1971) or changing their filtering selectivity (Palade & Barchi 1977; Furman & Barchi 1978). More recently, some chiral analogues of clofibric acid were found to reduce GCl with a potency that was dependent upon their enantiomeric configuration. Hence, the presence of a stereoselective receptor controlling the Cl^{-1} movements through specific channels has been proposed (Bettoni et al 1985, 1987). In the present study the effect of diphenylamine-2-carboxylate (DPC), a drug known to be a potent Cl^- channel blocker in the thick ascending limb segment of fish and mammalian nephrons (Wittner et al 1984; Di Stefano et al 1985; Wangemann et al 1986) was evaluated on the electrical properties of rat extensor digitorum longus (EDL) muscle fibres in-vitro.

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Materials and Methods

All experiments were carried out on male Wistar rats of 180–200 g. For in-vitro electrophysiological recordings, EDL muscles were removed, placed in a muscle bath maintained at 30 + 1°C and perfused with a physiological solution with or without the test compound.

The normal physiological solution had the following composition in mM: NaCl 148; KCl 4·5; $CaCl_2$ 2·0; $MgCl_2$ 1·0; $NaHCO_3$ 12 and glucose 5·5. The Cl^--free solution was made up by equimolar substitution of methylsulphate salts for NaCl and KCl and nitrate salts for $CaCl_2$ and $MgCl_2$. A Ca^{2+} reduced medium was obtained by lowering the $CaCl_2$ concentration from 2 to 0·5 mM and replacing the equimolar amount of Cl^- as NaCl and KCl.

The physiological solutions were bubbled with 95% O_2 and 5% CO_2 and the pH of the solutions with and without the test compound was adjusted to 7·3–7·4 (Conte Camerino et al 1985; Palade & Barchi 1977; Furman & Barchi 1978).

A diphenylamine-2-carboxylate stock solution of 10^{-4} g mL^{-1} was prepared in 1% sodium bicarbonate aqueous solution. The final concentrations (5–240 μM) were obtained by further dilutions in normal, Cl^--free, or Ca^{2+}-reduced solutions.

The muscles were incubated with the drug solutions for at least 30 min before electrophysiological recordings and on each preparation no more than three doses of drug were tested.

A pair of microelectrodes, one for recording potentials and the other for passing current, were used for making intracellular recordings of membrane potentials and cable properties (Camerino & Bryant 1976; Conte Camerino et al 1985).

The cable parameters determined in both normal and Cl^--free medium before and after the addition of the test drug, were calculated from the electrotonic potentials elicited by square wave hyperpolarizing current pulses and recorded at a distance of 0·05 mm and at about one space constant

from the current injecting electrode. From the input resistance, space constant and an assumed myoplasmic resistivity (Ri) of 125 ohm cm (Camerino & Bryant 1976) the fibre diameter, the membrane resistance, Rm and the total membrane capacitance were then calculated. The total membrane conductance Gm was 1/Rm in normal physiological solution and the potassium conductance GK was 1/Rm in Cl^--free medium. The mean Cl^- conductance, GCl, was calculated as the mean Gm minus the mean GK (Camerino & Bryant 1976; Conte Camerino et al 1985). From these determinations, it was possible to construct a dose-response curve for GCl of each preparation. Moreover, the resultant conductances versus concentration curves were fitted to a single site binding equation with a non linear least squares method, from which the IC50 $\pm$ s.d. (concentration required to half maximal GCl block $\pm$ standard deviation) was determined (De Coursey et al 1981).

The excitability characteristics of the sampled fibres, were determined intracellularly at different concentrations of the compound by observing the intracellular membrane potential response recorded from one microelectrode to a square-wave constant-current delivered by a second microelectrode inserted within 100 μM from the voltage electrode. In each fibre the membrane potential was set by a steady holding current to -80 mV, before passing the depolarizing pulses (Bettoni et al 1987). During some recordings of excitability, to avoid contraction of the fibres, dantrolene sodium was added to the bathing solution in a concentration of 2 mg L^{-1} (Adrian & Bryant 1974).

The data are expressed as mean $\pm$ s.e.m. Significance of differences between group means was calculated by paired or unpaired Student's t-test. The criterion for statistical significance was $P<0.05$. The estimates for s.e.m. of GCl were obtained from the variances of Gm and GK, assuming no covariance, using standard methods (Eisenberg & Gage 1969). Standard deviations for IC50 values were calculated from the variance-covariance matrix, obtained during non-linear procedures (De Coursey et al 1981).

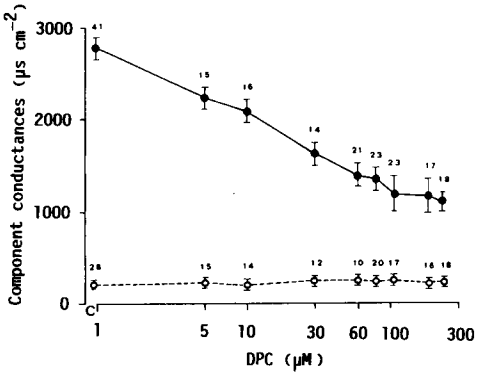

FIG. 1. Effect of diphenylamine-2-carboxylate (DPC) on the resting component conductances of rat EDL muscle fibres to chloride (GCl) (—●—) and potassium (GK) (- - ○ - -) ions. Each point represents the mean $\pm$ s.e.m. of several fibres (the number of fibres is noted on each point) from 3–10 muscles. The average GCl and GK before the addition of the drug was taken as control (C). At all DPC concentrations GCl was significantly different from the control value ($P<0.01$ or less), while GK was always unchanged with respect to the control value. The DPC doses are plotted on a logarithmic scale.

Results

In normal physiological solution, DPC caused a significant dose-dependent increase of membrane resistance (Rm). After the application of 240 μM of DPC, Rm reached a maximum value of 880 ± 131 ohm cm^2 ($n=18$) from a control value of 341 ± 21 ohm cm^2 ($n=41$). The drug did not affect the fibre diameter nor the specific membrane capacitance, even at the highest concentrations tested. In Cl^--free medium, DPC did not affect Rm. Rm was 4823 ± 780 ohm cm^2 in control fibres and 4252 ± 701 ohm cm^2 in the presence of 240 μM of DPC. The other variables measured were also unchanged by DPC in Cl^--free medium. Converting the Rm values to conductance showed that the increase of Rm by DPC observed in normal physiological solution was entirely explicable in terms of dose-dependent fall in GCl (Fig. 1). Over the whole concentration range tested DPC was without effect on GK. The DPC concentration required for 50% reduction (IC50 $\pm$ s.d.) of GCl was 120 ± 1.2 μM. This concentration was close to the maximal effective concentration.

DPC treatment did not affect the resting membrane potential of the tested fibres ($-78+2$ mV and -78 ± 8mV in control fibres and in fibres treated with 240 μM DPC, respectively). As would be expected from the reduction of GCl, the drug produced a dose-dependent increase in excitability (Fig. 2). This was characterized by a significant decrease of the current needed to evoke the first action potential (I_1) (Fig. 3A). The ratio between the threshold current and the current necessary to evoke the second action potential (I_1/I_2) increased dose-dependently, approaching unity at the highest concentrations tested (Fig. 3B). DPC significantly increased the delay (latency, Lat) between the onset of the current pulse, and initiation of the action potential (Fig. 3C) and it increased the number of action

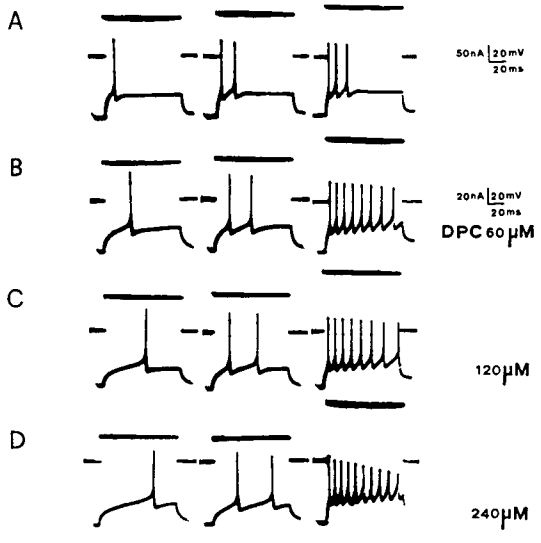

FIG. 2. Effect of diphenylamine-2-carboxylate (DPC) on intracellular action potentials of rat EDL muscle fibres: A in normal Ringer; B, C, D after addition of increasing concentrations of DPC to the bath. Upper trace, constant current pulse; lower trace, membrane potential. Initial resting potentials: -78 mV (A), -80 mV (B), -77 mV (D); membrane potential was set to -80 mV before passing the current pulse. Temperature 30°C.

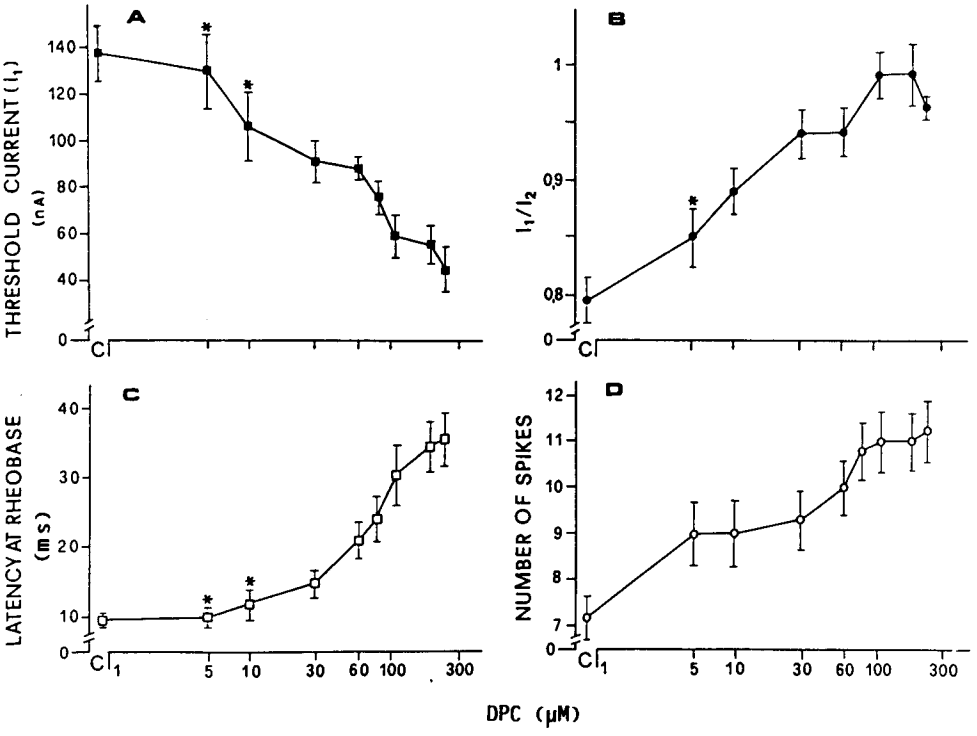

FIG. 3. Effect of diphenylamine-2-carboxylate (DPC) on excitability characteristics of rat EDL muscle fibres: (A—■—) on the current necessary to evoke the first action potential (threshold current I_1), (B—●—) on the ratio between the threshold current and the current to evoke the second action potential (I_1/I_2), (C—□—) on the latency at the rheobase of the action potential, (D—○—) on the maximum number of spikes elicited. Each point represents the mean ± s.e.m. of 15–33 fibres from 3–10 muscles. The average of each variable before the addition of the drug was taken as control (C). All points (except those with an asterisk*) of DPC dose-response curves are significantly different ($P < 0.01$ or less) from the corresponding control values. The DPC doses are plotted on a logarithmic scale.

potentials evoked by a standard stimulus (Fig. 3D). DPC did not affect the overshoot of the first action potential as would be expected for drug that has no effect on Na^+ conductance.

In another set of experiments the effect of DPC was evaluated in a low Ca^{2+} medium. This procedure is known to enhance the myotonic activity of some specific blockers of skeletal muscle's GCl, such as 9AC, by facilitating the appearance of self-sustaining action-potentials (Furman & Barchi 1978). However in low Ca^{2+}-medium, DPC did not cause any further increase of excitability nor was any self-sustaining repetitive activity produced. At least 30 min of incubation with DPC was necessary for the appearance of the effects of the drug on both GCl and excitability. The effects of DPC were slowly (30–60 min) but fully reversible.

Discussion

Recently DPC and some analogue compounds have been found to be potent and specific Cl^- channel blockers in the basolateral membrane of the thick ascending limb of rabbit and mouse nephrons (Wittner et al 1984; Di Stefano et al 1985; Wangemann et al 1986), and in the rectal gland tubules of the shark (Greger & Gogelin 1985).

Under our experimental conditions, the in-vitro application of DPC to rat EDL muscles caused a specific dose-dependent block of GCl, while GK was practically unaffected by the treatment. As a consequence of the decreased GCl, a significant increase of fibre excitability was observed.

Table 1. Drug acting on Cl^- channel conductance.

Tissue	DPC	9-AC	CPIB $S-(-)$
	IC50 (μM)		
Renal epithelia	30	100	
Skeletal muscle	120	20	10

Concentrations (μM) of diphenylamine-2-carboxylate (DPC), anthracene-9-carboxylic acid (9AC) and 2-*p*-chlorophenoxyisobutyric acid (CPIB, as the $S-(-)$ isomer) required to block GCl half-maximally in renal epithelia and skeletal muscle fibres of mammals.

Nevertheless, on skeletal muscle, DPC was less effective than other drugs known to be specific blockers of Cl^- channel conductance in striated muscle fibres. For instance, in our system 120 μM of DPC produced a half-maximal block of GCl; the same effect was exerted by 20 μM of 9AC (Bryant & Morales-Aguilera 1971; Furman & Barchi 1978).

Furthermore, we have recently shown that some chiral analogues of chlorophenoxyisobutyric acid can block muscle GCl stereospecifically, with an IC50 even lower than that of 9AC (Bettoni et al 1985; 1987) (Table 1). In accordance with the effects on GCl, the difference between DPC and 9AC can also be observed on excitability of striated muscle fibres. In fact, Furman & Barchi (1978) found that 50 μM of 9AC prolonged the latency of action potential by 400% whereas, during our experiments, a 200% increase of the same variable occurred with 240 μM of DPC. Furthermore, experiments

performed in a reduced Ca^{2+} concentration showed that DPC is unable to cause any self-sustaining action potential, yet this procedure predisposes to after discharge and enhances 9AC induced myotonic firing (Furman & Barchi 1978). It should be emphasized that Cl^- channels of skeletal muscle and of renal epithelia show a different affinity for Cl^- channel blockers (Table 1). For example, Di Stefano et al (1985) found that DPC blocks GCl in renal epithelium with an IC50 of 30 μM, and some structural analogues of DPC are even more potent (Wangemann et al 1986). On the other hand, 9AC has been found to be much less effective on renal epithelia than on skeletal muscle, with an IC50 of at least 100 μM. Some analogues of 9AC were practically ineffective (Wittner et al 1984; Wangemann et al 1986).

These observations support the idea of morphological, and consequently functional, differences of Cl^- channels between skeletal muscle and renal epithelia. Nevertheless, it would not be surprising if further studies show that the different membrane proteins transporting Cl^- have parts of the molecule in common. In this connection, it may be noted that in recent experiments designed to clarify the behaviour of Cl^- channels in airways epithelia from normal subjects and from patients with cystic fibrosis, DPC decreased Cl^- channel conductance in both instances (Welsh & Liedtke 1986). The observation that the inhibitory potency of several drugs on muscle GCl depends strictly upon their molecular structure, gives support to the concept of a receptor-type binding site that governs channel activity.

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