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SHORT COMMUNICATIONS

3,4-Diaminopyridine partially reverses phospholipid breakdown induced by oxygen deprivation of cultured myocardial cells

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Ischemia in heart tissue induces complex perturbations in the control of cellular ion homeostasis which result in a loss of K^+, a rise of cell Na^+ and an increase in cytosolic free Ca^{2+} (for reviews see [1, 2]). These alterations could be linked to another consequence of ischemia: the degradation of phospholipids, which has been observed *in vivo* in the heart [3–5] as well as in cultured heart muscle cells [6]. In fact, phospholipid depletion is thought to be due to the activation of endogenous phospholipases [4], sustained by the progressively increasing Ca^{2+} overload [1, 3, 7] following ischemia; and to the enhanced sensitivity of anoxic sarcolemma to phospholipases as the ATP concentration falls [8].

On the other hand, phospholipids are probably involved in the sarcolemmal binding of Ca^{2+} [9] and in the control of ion fluxes [10, 11], and they could be structural components of ion channels [12]. Thus their depletion could be responsible for the loss of the selective membrane permeability to the ions.

Aminopyridines, which are known to facilitate transmitter release at autonomic and central chemical synapses, appear to selectively block K^+ channels in excitable membranes (for reviews, see [13–15]). They exhibit positive inotropic activities, in isolated hearts [16, 17], probably by increasing Ca^{2+} availability to the contractile fibers and/or by inducing a release of endogenous adrenaline [17]. Recently, Peterson and Gibson [18] showed that 3,4-diaminopyridine counterbalanced the effects of hypoxia on Ca^{2+} movement in brain synaptosomes. This observation encouraged us to pursue our search for a possible effect of this molecule in another experimental model, i.e. cultured heart cells. As published previously [6], ischemia is mimicked in this model by the imposition of several consequences of flow-arrest upon the cells: exhaustion of energy-yielding substrates, accumulation of metabolic end-products, and oxygen deprivation. We observed that phospholipids were progressively depleted in muscle cells (M), but not in non-muscle cells (F). In the present paper, we show that 3,4-diaminopyridine (DAP) partly reverses the phospholipid breakdown occurring in M cells.

Methods

The methods used have been detailed previously [6]. A single cell suspension was obtained by trypsinization of hearts excised from 3-day-old Sprague–Dawley rats. Muscle cells (M) were separated from non-muscle cells (F) by means of a differential attachment technique and the proliferation of the residual F cells in M cell cultures was limited by the addition, 48 and 72 hr after plating, of arabino-furanosyl-cytosine at a final concentration of 10^{-6} M. Experiments were performed with 6-day-old cultures, with the cells remaining in the medium in which they were grown. M culture flasks were divided into 4 equal groups: the 1st group consisted of normally oxygenated cells, the 2nd of normally oxygenated cells treated with DAP, the 3rd of oxygen deprived cells, and the 4th of oxygen deprived cells treated with DAP. Groups 1 and 2 were gassed with air + CO^2 (N_2 75% + O_2 20% + CO_2 5%), groups 3 and 4 were gassed with nitrogen + CO_2 (95% + 5%) for 55 min at 37°. The final pO_2 of the culture medium was 115 ± 6 mm Hg for groups 1 and 2, 25 ± 6 mm Hg for groups 3 and 4. Oxygen deprivation was maintained for 3 hr in the first series of experiments, and for 20 hr in the second series. DAP was dissolved in a phosphate buffered salt solution and a final concentration of 10^{-4} M was used. DAP was added to the appropriate flasks (groups 2 and 4) 2 hr before the end of the experiment, thus allowing us to compare its effects on oxygen deprivation in both an early stage and a later one, when phospholipid alterations are more pronounced.

The flasks were chilled, and cells were removed by scraping and homogenized in buffered salt solution. Cellular protein content was assayed according to Hartree [19]. The lipids, extracted according to Folch *et al.* [20], were fractionated by silicic acid column chromatography; neutral lipids, eluted with hexane/ether 90:10 (v/v) [21] were discarded; polar lipids, eluted with 100% methanol [22] were dried and stored at −20° under nitrogen until assayed for fatty acid composition. The phospholipid residue, redissolved in 100% methanol, was subjected to BF_3 transmethylation after the addition of heptadecanoic acid as an internal standard. Fatty acid methyl esters were extracted with 100% hexane and analyzed by means of a gas chromatograph (Carlo Erba 4100) equipped with a capillary column and connected to an integrator (Spectra Physics). Among the 15 fatty acids identified, those representing <1% of the total mass were not taken into account: they were C 18:3, C 20:0, C 20:1, C 20:3, C22:0, C 22:1 and C 24:0. The results, expressed as percentages of the total amount and as μg/mg of protein, were subjected to variance analysis [23].

Results

For group 1, i.e. air-gassed M cells, the total amounts and the individual quantities of phospholipid fatty acids were similar for both time periods considered. Under oxygen deprivation they decreased progressively. The total amount (Fig. 1) was reduced by 32% after 3 hr, and by 47% after 20 hr of incubation. The decrease was only significant for arachidonic acid ($P < 0.01$) for the shorter time period and the proportions of the fatty acid moieties, expressed as percentages of the total mass, were not modified. After 20 hr of incubation (Table 2), the loss of the individual fatty acids was most pronounced and affected the poly-unsaturated fatty acids most markedly (−70 to −80% vs −46% for stearic acid, and −20 to −30% for the other acids). As a consequence, fatty acid ratios were altered. With the exception of the proportion of stearic acid, which remained constant, the percentages of saturated, mono- and dienoic fatty acids were enhanced, while the percentages of the polyunsaturated fatty acids were drastically reduced.

In both series of experiments, DAP had no significant effect on normally oxygenated cells. However, when added to cultures deprived of oxygen for 3 hr (Fig. 1, Table 1), it almost completely restored the absolute amounts of phospholipid fatty acids. When present for the last 2 hr of the 20 hr experiments, DAP partly counteracted the phospholipid breakdown observed following oxygen deprivation: it enhanced the total phospholipid content (Fig.

1) above the values found in oxygen deprived cells ($P < 0.01$), but did not restore the air values ($P < 0.01$). The individual fatty acid quantities were unequally affected (Table 2): saturated fatty acids were restored to the same extent as the total amount, mono- and dienoic fatty acid contents were completely reestablished, so that they no longer differed from air values. On the contrary, poly-unsaturated fatty acids were poorly salvaged: arachidonic amounts remained closer to oxygen deprived values than to air values ($P < 0.01$ in both cases); docosahexaenoic acid levels remained on a par with nitrogen deprived values ($P < 0.01$ vs. air values). Consequently, the percentage distribution most closely resembled that of oxygen deprived M cells.

Discussion

The phospholipid breakdown observed in cultured M cells subjected to oxygen deprivation resembles that observed by Chien in the ischemic myocardium *in situ* [3]. The major loss of poly-unsaturated fatty acids underlines the preferential attack on the phospholipids esterified with these fatty acids and/or on the number 2 position of the glycerol backbone. It is tempting to assign this degradation to phospholipase A_2 activation. However, unlike Chien [3], we did not find any accumulation of non-esterified arachidonic acid. The total amount of non-esterified fatty acids was enhanced by 33% [24] in oxygen deprived cells; but the arachidonic acid percentage decreased from 22 to 9% [25], and as a consequence, the amount of arachidonic acid was reduced to half of that observed in normally oxygenated cells. Moreover, the modifications observed in the percentage distribution of fatty acids suggest a phospholipid remodeling through the preferential reinsertion of palmitoleyl-, oleyl- and linoleyl-moieties. Thus, at present, the metabolic pathways followed by the missing arachidonic acid remain unclear and require further investigations. In particular, it will be necessary to examine the conversion of arachidonic acid into prostanoids.

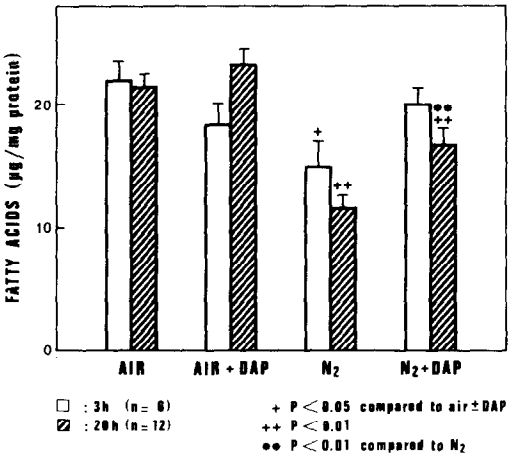

Fig. 1. Effect of 3,4-diaminopyridine (DAP, 10^{-4} M) on total phospholipid fatty acid content of heart muscle cells.

DAP partly reversed the phospholipid depletion induced by oxygen deprivation, without affecting the disproportion found between the more or less saturated fatty acids. Interpreting these data in an attempt to clarify the underlying mechanisms, some hypotheses can be proposed and others eliminated. DAP could interfere directly with phospholipases. This is suggested by the stoichiometric effect of this molecule: given for 2 hr in both experimental series, DAP salvaged equal quantities of phospholipid fatty acids, i.e. 5.2 µg/mg of protein of the total amount, 1.58 of stearic acid, 0.45 of linoleic acid, 1.47 of arachidonic acid and 0.13 of docosahexaenoic acid. DAP could limit phospholipase activation by impeding Ca^{2+} overload in heart cells. It may act directly on calcium movements, or indirectly by blocking the K^+ efflux. An effect on acyltransferase activities is unlikely, since DAP did not interfere in either the defective reacylation of arachidonic acid, or the preferential reacylation of mono- and dienoic fatty acids.

DAP improvement of ion homoeostasis through the restoration of high-energy phosphates can be excluded: some experiments showed that DAP did not prevent ATP and phosphocreatine losses during oxygen deprivation.

A facilitating effect of the Ca^{2+} dependent release of neurotransmitters, as suggested in hypoxic brain synaptosomes [26], could hardly be implicated: adrenergic and muscarinic receptors are known to be present and functional in heart cell cultures; however, myocytes are thought to be noninnervated *in vitro* [27].

In summary, although ineffective on normally oxygenated heart cells in culture, DAP partly reversed the

Table 1. Effect of 3,4-diaminopyridine (DAP, 10^{-4} M) on heart muscle cells (M) subjected to oxygen deprivation for 3 hr

Fatty acids	Phospholipid fatty acids (µg/mg protein)				Phospholipid fatty acids (% distribution)			
	Air	Air + DAP	N_2	N_2 + DAP	Air	Air + DAP	N_2	N_2 + DAP
C14:0	0.26 ± 0.04	0.24 ± 0.06	0.28 ± 0.03	0.22 ± 0.01	1.1 ± 0.1	1.3 ± 0.3	1.9 ± 0.3	1.1 ± 0.1
C16:0	4.04 ± 0.43	2.96 ± 0.44	2.61 ± 0.47	3.65 ± 0.35	18.1 ± 1.0	16.1 ± 1.2	17.4 ± 0.9	18.0 ± 1.0
C16:1	0.62 ± 0.11	0.48 ± 0.07	0.57 ± 0.08	0.66 ± 0.12	2.9 ± 0.4	2.6 ± 0.5	3.9 ± 0.3	3.3 ± 0.6
C18:0	6.21 ± 0.50	5.08 ± 0.55	3.96 ± 0.77	5.53 ± 0.59	28.2 ± 1.6	27.6 ± 0.7	26.3 ± 2.0	27.3 ± 1.5
C18:1	2.00 ± 0.28	1.79 ± 0.12	1.58 ± 0.15	1.89 ± 0.21	9.1 ± 0.9	9.7 ± 1.1	10.3 ± 1.2	9.4 ± 1.1
C18:2	1.92 ± 0.52	1.81 ± 0.26	1.62 ± 0.20	2.09 ± 0.34	8.6 ± 0.9	9.8 ± 1.0	10.7 ± 0.6	10.4 ± 1.5
C20:4	5.83 ± 0.33	5.04* ± 0.34	3.55‡ ± 0.39	5.06* ± 0.34	26.5 ± 1.2	27.3 ± 1.5	23.8 ± 1.0	25.1 ± 1.5
C22:6	0.81 ± 0.15	0.77 ± 0.04	0.58 ± 0.06	0.70 ± 0.10	3.8 ± 0.6	4.2 ± 0.9	4.0 ± 0.5	3.6 ± 0.6

Air = controls; N_2 = oxygen deprived cells. DAP was added for the last 2 hr of the experiment.
Each value represents the mean ± S.E.M. for six experiments.
* $P < 0.05$ compared to oxygen deprived cells.
‡ $P < 0.01$ compared to air ± DAP.

Table 2. Effect of 3,4-diaminopyridine (DAP, 10^{-4} M) on heart muscle cells (M) subjected to oxygen deprivation for 20 hr

Fatty acids	Phospholipid fatty acids (μg/mg protein)				Phospholipid fatty acids (% distribution)			
	Air	Air + DAP	N_2	N_2 + DAP	Air	Air + DAP	N_2	N_2 + DAP
C14:0	0.12 ± 0.02	0.15 ± 0.03	0.19 ± 0.03	0.28† ± 0.05	0.6 ± 0.1	0.7 ± 0.1	1.8‡ ± 0.3	1.7† ± 0.4
C16:0	3.55 ± 0.15	3.78 ± 0.25	2.47‡ ± 0.26	3.08 ± 0.21	16.3 ± 0.5	16.4 ± 0.6	21.1‡ ± 1.0	18.3 ± 1.1
C16:1	0.49 ± 0.06	0.51 ± 0.03	0.33 ± 0.05	0.50 ± 0.07	2.3 ± 0.3	2.2 ± 0.1	2.9 ± 0.4	3.1 ± 0.6
C18:0	6.24 ± 0.40	6.68 ± 0.40	3.39‡ ± 0.37	4.98 ± 0.50	28.6 ± 0.8	29.1 ± 0.7	29.1 ± 1.0	29.6 ± 1.1
C18:1	2.39 ± 0.09	2.61 ± 0.19	1.71‡ ± 0.14	2.35** ± 0.16	11.0 ± 0.7	11.4 ± 0.7	14.8‡ ± 0.4	14.0† ± 0.7
C18:2	1.50 ± 0.07	1.55 ± 0.11	1.16† ± 0.08	1.58** ± 0.09	7.0 ± 0.4	6.7 ± 0.4	10.0† ± 1.1	9.4† ± 0.6
C20:4	6.11 ± 0.39	6.38 ± 0.45	2.01‡ ± 0.24	3.43‡** ± 0.52	28.1 ± 0.9	27.5 ± 1.0	17.2‡ ± 0.7	20.4‡* ± 0.7
C22:6	1.14 ± 0.11	1.13 ± 0.14	0.25‡ ± 0.05	0.39‡ ± 0.09	5.2 ± 0.4	4.8 ± 0.5	2.2‡ ± 0.3	2.3‡ ± 0.3

Air = controls; N_2 = oxygen deprived cells. DAP was added for the last 2 hr of the experiment. Each value represents the mean ± S.E.M. for 12 experiments.
* $P < 0.05$ and ** $P < 0.01$ compared to oxygen deprived cells.
† $P < 0.05$ and ‡ $P < 0.01$ compared to air ± DAP.

phospholipid cleavage induced in muscle cells (M) by oxygen deprivation. Added for 2 hr after 1 or 18 hr of oxygen deprivation, it salvaged identical amounts of phospholipid fatty acids. These data suggest a reduced phospholipase activation, perhaps resulting from an improved ion homoeostasis.

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