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The nature of DT-diaphorase (EC 1.6.99.2) activity in plasma membrane of astrocytes in primary cultures

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This is the confirmation of an earlier indication (Mersel, M., Malviya, A.N., Hindelang, C. and Mandel, P. (1984) Biochim. Biophys. Acta 778, 144–154) that the plasma membrane of astrocytes in primary cultures is endowed with DT-diaphorase (EC 1.6.99.2) activity. It is observed that the NADPH-2,6-dichloroindophenol diaphorase activity found in the isolated plasma membrane is not inhibited by dicoumarol. DT-diaphorase-type activity is also observed on the cell surface employing dichloroindophenol as external electron acceptor and it is found to be a dicoumarol-sensitive NADH dehydrogenase.

Introduction

It seems well founded that the plasma membrane [1] (irrespective of its origin) possesses its own redox system distinct from the extensively characterized redox system of microsomal or mitochondrial membrane [2]. The knowledge of oxidoreductase enzyme system located in the plasma membrane has emanated primarily from the study of substrate and acceptor specificity. Definitive correlation between a number of acceptor oxidoreductases activity of the plasma membrane, isolated from a variety of cells (eukaryotic or prokaryote) will await the actual isolation of the enzyme. Ever since the discovery of DT-diaphorase system by Ernster et al. in 1962 [3] most of the studies have been reported on the soluble enzyme, although recently [4] NADPH-diaphorase activity is described in the human neutrophils particulate fractions. During the writing of this manuscript a report has appeared [5] purifying NADH-diaphorase activity from the membrane bound NADPH oxidoreductase of neutrophils. After following the first indication [6] of DT-diaphorase enzyme located in a plasma membrane, the present report deals with definite localization of this redox system in the plasma membrane of astrocytes primary cultures. It is further delineated here that the NADPH-DCIP diaphorase located in the isolated plasma membrane, unlike cytosolic enzyme, is insentitive to dicoumarol. The relative merit of dicoumarol sensitivity of the oxidoreductase system when assayed in the isolated plasma membrane as contrasted from the enzyme activity assayed at the cell surface in intact system is evaluated. A preliminary report of this work has been presented [7].

Experimental procedures

Cell cultures. Astrocytes primary cultures derived by mechanically dissociated brain hemispheres of neonatal rats were obtained according to Sensenbrenner et al. [8], except that the culture medium was Dulbecco's Modified Eagle Medium (DMEM) (Gibco, U.S.A.). The cells were seeded at a density of $1.5 \cdot 10^6$ cells per 35 mm diameter culture dishes (Falcon, U.S.A.) kept in 5% CO_2

atmosphere at 37°C, growth medium was changed at 5 days intervals with DMEM-containing 10% fetal calf serum (Flow Laboratories, U.S.A.).

Assay of cell surface NADH or NADPH dichloroindophenol reductase activity. Two-weeks-old cell cultures were rinsed five times with phosphate buffer (50 mM, pH 7.2), 3.5 ml phosphate buffer was added to the washed cells and 30 μl of dichloroindophenol (100 μM final concentration). By gently shaking the culture dish, the dye was diffused throughout the culture surface evenly. The reaction was started by adding 10 μl of NADH or NADPH (50 μM final concentration) to the medium and the absorbance was recorded at 600 nm at each two minutes interval by withdrawing 500 μl aliquots from the Petri dish.

Effect of antimycin, rotenone and dicoumarol on cell surface NADH or NADPH dichloroindophenol reductase activity. NADH or NADPH dichloroindophenol reductase activity was determined as described above, except that 10 μl of dicoumarol (prepared in phosphate buffer, pH 7.2, 3 μM final concentration), 10 μl of rotenone (prepared in ethanol, 3 μM final concentration) or 10 μl of antimycin (prepared in ethanol, 1 μM final concentration) were added individually and concomitantly with DCIP.

Cell viability. After each assay, the cultures were observed under a phase contrast microscope and the cell viability assessed by the Trypan blue dye exclusion method.

Amount of dichloroindophenol associated with the cell as a function of incubation time. Two-weeks-old cell cultures were rinsed five times with phosphate buffer and incubated with 3.5 ml of the same buffer containing 60 μg DCIP. At the interval of 1, 2, 4, 5, 10 and 13 min the incubation medium was discarded, the cells were harvested and extracted twice with 1 ml ethanol. Aliquots of the ethanol extract were dried under a nitrogen stream, the residue dissolved in 10 μl ethanol, spotted on Silica gel coated plates and eluted with methanol/ammonia (1.5 M) (100 : 18. v/v). The chromatogram was recorded by a Cammag MC scanner at 542 nm and the peak area was integrated. Calibration experiments showed that the area was linear between 0.1 and 2.0 μg of chromatographed DCIP.

Preparation of the cytosolic fraction. Cell cultures were rinsed twice with phosphate buffer, harvested in 0.32 M sucrose containing 2.0 mM mercaptoethanol and were homogenized in a Elvenjehm potter. The cellular homogenate was centrifuged at $900 \times g$ for 10 min, the resulting supernatant was centrifuged at $100\,000 \times g$ for 1 h and the resulting supernatant was used as a cytosolic fraction. Cellular homogenate and plasma membranes were prepared as described previously [6].

NADH or NADPH acceptor oxidoreductase (DCIP) activity. This was assayed using 1 ml of the cytosolic fraction or plasma membranes, appropriately diluted in 1 ml phosphate buffer. The oxidation of NADH or NADPH was monitored at 600 nm. The concentration of substrates, inhibitors and cofactors was similar to that described for the cell surface enzymatic assay. Proteins were determined according to Lowry et al. [9] using bovine serum albumin as standard.

Results

Table I outlines the acceptor oxidoreductase activity in cell homogenate and cytosol: the identical activity observed utilizing exogenous NADH or NADPH is indicative of DT-diaphorase system. The cytosolic DT-diaphorase activity was sensitive to dicoumarol as was the activity observed in the cell homogenate. Some slight inhibition by antimycin or rotenone, the well-known inhibitors of mitochondrial electron transport at site II or site I, respectively, has been seen in the cytosolic enzyme. However, none of these inhibitors influenced significantly the NADH or NADPH-DCIP oxidoreductase activity in the isolated plasma membrane.

The data obtained when the oxidoreductase activity was assayed at the cell surface in intact cell are intriguing (Table II). In marked distinction from the isolated plasma membrane, the cell surface enzyme failed to utilize exogenous NADPH. Cell surface NADH-DCIP-reductase was inhibited by dicoumarol analogous to the dicoumarol-sensitive nature of diaphorase system [3]. These results are expressed either as nmol/min per mg cellular proteins or as nmol/min per mg plasma membrane proteins, since the latter represents 1% of the total cellular proteinaceous material [6]. The data expressed as nmol/min per

TABLE I

OXIDOREDUCTASE-SPECIFIC ACTIVITY IN THE CELLULAR HOMOGENATE AND IN THE CYTOSOL WITH DCIP AS ELECTRON ACCEPTOR

The activities are expressed as nmol/min per mg protein. The final concentrations of rotenone, antimycin and dicoumarol were 3, 3 and 1 μM, respectively; those of NADH and NADPH were 100 μM and that of DCIP was 100 μM. The percentages of enzymatic activity inhibited are expressed in brackets. Results are expressed as means of ±S.D. Cell homogenate, four experiments; cytosol, three experiments.

	Cell homogenate		Cytosol	
	NADH	NADPH	NADH	NADPH
Control	15.0 ±0.5	15.0 ±0.5	48.0 ±4.0	42.0 ±2.8
Rotenone	15.0 ±0.5	14.0 ±0.5	36.0(25) ±1.0	39.0(7) ±1.0
Antimycin	15.0 ±0.5	15.0 ±0.5	40.0(16) ±2.5	33.0(21) ±3.2
Dicoumarol	0.35(98) ±0.05	0.46(97) ±0.07	9.8(80) ±1.5	3.9(91) ±0.5

mg membrane proteins correlated with that of the isolated plasma membranes and depicts the activities localized at the cell surface. This is further supported by the design of experiment illustrated in Fig. 1. Here the cells were incubated in 35 mM petri dishes with dichloroindophenol and after initial equilibration (about 5–6 min) when no more nonspecific change in absorption at 600 nm was seen, NADH or NADPH were added to the medium. At each 2 min intervals 500 μl of the supernatant was withdrawn. The same experiment was repeated in the presence of individual inhibitors mentioned. It does seem obvious that NADH-linked diaphorase activity was sensitive to dicoumarol and NADPH was not able to donate electrons to the external acceptor.

Proper control was done to ensure that the reduction of DCIP was an actual phenomenon and not an artifact of its penetration into the cell interior or exterior. Removal of the reaction mixture after 13 min incubation showed that only 1% of DCIP remained associated with the cellular material. Therefore, it can be said that the activity of the enzyme assayed was actually the activity present at the cell surface. Furthermore, the observed DCIP reduction was not due to any extracellular component released, since incubating the culture medium with DCIP and addition of either NADH or NADPH did not reveal any reductase activity.

TABLE II

OXIDOREDUCTASE-SPECIFIC ACTIVITY IN THE ISOLATED PLASMA MEMBRANES AND AT THE CELL SURFACE WITH DCIP AS ELECTRON ACCEPTOR

The final concentrations of rotenone, antimycin and dicoumarol were 3, 3 and 1 μM, respectively, NADH and NADPH were 100 μM and that of DCIP 50 μM. For cell surface in intact cells, the activity was assayed at the cell surface of two-week-old astrocytes primary cultures. Details of the procedure are described in Experimental Procedures. The results are expressed as mean ± standard deviation of four experiments. No NADPH oxidoreductase activity was seen at the cell surface.

	Isolated plasma membrane		Cell surface in intact cells	
	NADH (nmol/min per mg plasma membrane protein)	NADPH (nmol/min per mg plasma membrane protein)	NADH (nmol/mg per cellular protein)	NADH (nmol/mg per plasma membrane protein)
Control	69.0 ±4.0	60.0 ±3.0	1.0 ±0.05	100 ±8.0
Rotenone	66.7(3) ±4.0	57.0(5) ±2.8	1.1 ±0.06	110 ±9.5
Antimycin	67.2(3) ±4.0	57.6(5) ±2.8	0.9(10) ±0.04	90 ±7.5
Dicoumarol	55.2(20) ±2.7	52.8(12) ±2.5	0.3(70) ±0.02	30 ±2.0

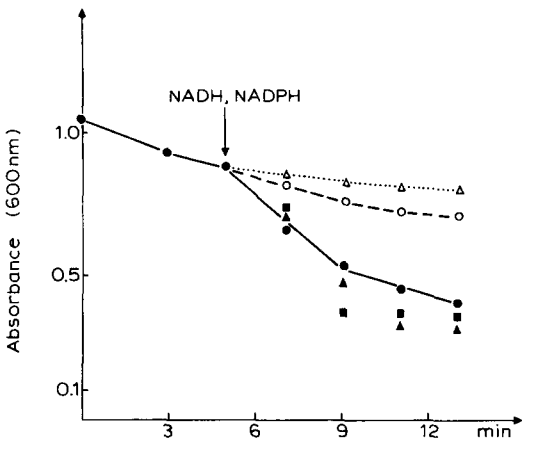

Fig. 1. NADH and NADPH-2,6-dichloroindophenol diaphorase activities detected at the surface of two-weeks-cultured neonatal rat astrocytes. ●——●, NADH, control; ■——■, NADH, antimycin; ▲——▲, NADH, rotenone; ○——○, NADH, dicoumarol; △——△, NADPH, control. After washing the cultures (as described in the Experimental Procedures section) dichloroindophenol (100 μM final concentration) was added and it was diffused throughout the culture surface, evenly, by gently shaking the culture dish. The reaction was initiated by adding 50 μM of NADH or NADPH to the medium and absorbance was recorded at 600 nm at each two minutes interval by withdrawing 500 μl aliquots.

Discussion

A definite location of DT-diaphorase activity in the astrocytes plasma membrane either isolated or on the cell surface of intact cell is the thrust of this manuscript. As pointed out earlier [6] it is further confirmed that DCIP is the favoured external electron acceptor for the astrocytes plasma membrane diaphorase system. It is also observed here that the DCIP may be used as an impermeant (an analogy with ferricyanide) ion and about 1% of DCIP seems to associate with the cellular material. It does not in any way affect the assay of DT-diaphorase activity having DCIP as an external electron acceptor. The impermeant nature of NADH or NADPH either to the cell membrane [10,11] or to the mitochondrial membrane is very well established [12], and thus the reduction of the surrounding electron acceptor is the true index of the activity of a transmembrane dehydrogenase system located on the cell surface. The enzyme in the isolated plasma membrane is not sensitive to dicoumarol – a classical inhibitor of DT-diaphorase system. However, under the present experimental conditions DT-diaphorase activity assayed either in astrocytes cell homogenate or cytosolic fraction was, as expected, inhibited by dicoumarol. This clearly demonstrates that the insensitive nature of DT-diaphorase system towards dicoumarol assayed in the isolated plasma membrane is a characteristic of this redox system and not an artifact. Furthermore, when the DT-diaphorase activity was assayed on the cell surface in intact cell dicoumarol sensitivity of NADH-dichloroindophenol reductase activity was observed. The study on intact cell surface manifested only NADH-diaphorase system, while NADPH-diaphorase activity was totally devoid at the cell surface. The distinction observed between the diaphorase activity assayed in isolated plasma membrane when compared with the similar activity on the cell surface may be attributed to a number of possibilities. One such possibility may be that during plasma membrane isolation there occurs enzymatic modification thereby, dissipation of dicoumarol sensitivity. Secondly, the orientation of DT-diaphorase is entirely different in intact cell as contrasted from the isolated plasma membrane. In this context it may not be out of place to record that glucagon stimulates NADH dehydrogenase activity in isolated membranes while inhibiting this activity in perfused liver cells [1]. Here again a different enzyme active in the intact cell or enzyme modification occurring during membrane isolation may be implicated. A total absence of NADPH linked dehydrogenase activity on the cell surface and NADH-diaphorase activity sensitive to dicoumarol does support such a contention. The study reported here is the first study addressing the question of DT-diaphorase system of plasma membrane isolated from primary cultures of a nerve cell. This is of particular relevance in view of contemporary studies providing definite correlation between plasma membrane redox system and vital cellular functions such as: (a) growth control [13], (b) rise in intracellular pH [14,15], (c) divalent cations enhancing superoxide production [16], and (e) tumor promoting *onc* genes controlling specific protein kinases [17].

This study of DT-diaphorase-type activity observed in astrocytes in situ points out a character-

istic nature of glial cells which seems to assume importance only recently [18]. It has been lately realized that glial cells are of great importance in understanding the vexed question of information processing in nervous systems [19].

The localization of DT-diaphorase type system at the cell surface of astrocytes is of special relevance, since the role of this particular redox system in mediating cytotoxic [20,21] response is just beginning to emerge.

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