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PURIFICATION OF PLASMA MEMBRANES FROM MOUSE PAROTID GLAND AND MEMBRANE REORGANIZATION IN RESPONSE TO ISOPROTERENOL

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Two highly purified plasma membrane fractions have been obtained from mouse parotid glands by a combination of differential centrifugation and isopycnic centrifugation in discontinuous sucrose gradients. The membranes were characterized by enzymic, chemical and morphological criteria. The effect of isoproterenol, which induces parotid acinar cells to proliferate, upon sialic acid and five different enzyme activities located in the plasma membrane phosphodiesterase (EC 3.1.4.1), Mg^{2+}-ATPase (EC 3.6.1.4), leucine aminopeptidase (EC 3.4.1.1), protein kinase (EC 2.7.1.37) and sialyltransferase (EC 2.4.99.1), were quantified along the cell cycle. Plasma membrane sialic acid content falls 30% within 30 min and remains depressed for at least 6 h with the major restoration towards normal levels occurring between 12 and 16 h later. In contrast multiple daily isoproterenol injections lead to a more than 2-fold elevation of sialic acid content. Sialyltransferase activity rises 2-fold by 12 h after isoproterenol treatment and then rapidly falls. This enzyme has a pH optimum of 6.5, requires a divalent cation for activity and is inhibited by Triton X-100. Other enzyme activities showed markedly different changes after isoproterenol stimulation, either increasing, decreasing or remaining unaltered. These continuous functional modifications suggest an active role of the plasma membrane in the control of the proliferative cycle.

Introduction

The mouse parotid gland is a very homogeneous tissue, serous in nature, and constituted almost 90% by volume of acinar cells. The rodent parotid has been extensivley used for studying physiological processes such as stimulus-secretion coupling [1], DNA synthesis [2,3] and hypertrophy [4,5]. All of these events are induced by the synthetic β-adrenergic agonist isoproterenol which is believed to exert all its effects through interaction with specific receptors at the cell surface [6]. With regard to cell proliferation, the participation of the plasma membrane has been associated either to the synthesis of cyclic AMP [7,8] or to increased permeability to calcium ions [9,10] in a wide variety of systems. Usually these functional changes are related to specific structural changes at the plasma membrane which are based upon the structurally dynamic properties of this macromolecular aggregate [11]. These specific structural-functional changes at the plasma membranes might be associated directly or indirectly to many other specific intracellular processes [12]. In the mouse parotid

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Abbreviations: Mes, 2-(*N*-morpholino)ethanesulphonic acid; Mops, 3-(*N*-morpholino)propanesulphonic acid.

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chemical changes in the cell membrane have been implicated in the control of cell proliferation [13–15].

Although a simple subcellular fractionation of the mouse parotid has been described [16] a more extensive purification of the plasma membrane was required for further studies on the role of this membrane in controlling the various physiological responses to isoproterenol. In the present study a method for the purification of the plasma membrane is described and this preparation characterized. We have then analyzed five different enzyme activities and sialic acid content at various times after isoproterenol treatment, as indicative molecules of the plasma membrane functional state along the proliferative cycle. Taken together these results show that stimulation of parotid acinar cells with isoproterenol is followed by a major change or reorganization at the plasma membrane.

Materials and Methods

Animals. Male mice of the Porton strain, bred in the laboratory, and weighing 28 ± 3 g were used in all experiments. The mice were maintained on a 12-h light and dark schedule and fed ad libitum until 2 h before the experiment when food was withdrawn. DNA synthesis was induced by intraperitoneal injection in isotonic saline of 0.4 μmol DL-isoproterenol per g of body weight.

Tissue fractionation. Mouse parotid glands were dissected from control or isoproterenol-stimulated mice, excised from fat and lymph nodes and placed into isotonic buffer at 4°C. All further procedures were carried out at 4°C. The glands from 15–20 mice were pooled, finely minced with a razor blade and homogenized in 20 ml 0.32 M sucrose in buffer A (50 mM Tris-HCl (pH 7.4 at 20°C)/25 mM KCl/3 mM $MgCl_2$/2 mM $CaCl_2$) in a teflon/glass Potter-Elvehjem homogenizer. More than 80% of the acinar cells are broken and are separated from the larger material, mainly connective tissue, by passing through a double layer of cheesecloth.

Plasma membranes were isolated by differential and isopycnic centrifugation. Differential centrifugation was carried out according to a previously published scheme [16] except that the final step was a $3 \cdot 10^6$ $g \cdot$ min centrifugation. The pellet from this centrifugation was resuspended in 0.32 M Sucrose-buffer A and repelleted at the same speed. The resulting material is resuspended in 15 ml 1.31 M sucrose-buffer A and overlaid with 10 ml each of 1.11 M and 0.32 M sucrose-buffer A. After centrifugation for 16 h at 25000 rpm in the SW-27 rotor (Beckman Instrument, Palo Alto, CA) plasma membranes are recovered from the interphases of 0.32 M/1.11 M and 1./11 M/1.31 M sucrose-buffer A, diluted with 50 mM Tris-HCl (pH 7.4) and centrifuged for 10^7 $g \cdot$ min.

Analytical assays. Sialic acid was measured using a micro-method adapted from the Warren technique [17]. Protein was determined by the procedure of Bramhall et al. [18] with pre-treatment of the samples with NaOH [19]. DNA synthesis was quantitated by [^{3}H]thymidine incorporation as described by Durham et al. [3]. Chemical determination of cholesterol, phospholipid, RNA, DNA and carbohydrates were performed as previously described [16]. All determinations were in duplicate, except for sialic acid which was determined in triplicate.

Enzyme assays. Protein kinase (EC 2.7.1.37) activity was determined in the presence and absence of 20 μM cyclic AMP in a medium containing 13 mM $MgCl_2$, 1.5 mM dithiothreitol, 13 mM NaF, 0.45 mM [γ-^{32}P]ATP (20000 dpm/pmol), 5 mg/ml mixed histone and 20 μg plasma membrane protein; final volume 120 μl. After incubation for 5 min at 20°C the ^{32}P-labeled histone was quantitated as described by Corbin and Reimann [20] with duplicate determinations on duplicate assays. Mg^{2+}-ATPase (EC 3.6.1.4) was assayed in a medium containing 3 mM $MgCl_2$, 2.5 mM ATP (Tris form), 50 mM Tris-HCl (pH 7.4) and 25 μg protein. Phosphate released during a 30-min incubation at 37°C was quantitated according to Stanton [21]. Alkaline phosphatase (EC 3.1.3.1), phosphodiesterase (EC 3.1.4.1), 5′-nucleotidase (EC 3.1.3.5), glucose-6-phosphatase (EC 3.1.3.9) and succinate dehydrogenase (EC 3.1.99.1) were assayed as previously described [16]. Leucine aminopeptidase (EC 3.4.1.2) activity was evaluated according to Goldberg and Rutenburg [22] using L-leucyl-β-naphthylamide as substrate. Sialyltransferase (EC 2.4.99.1) activity was determined by measuring the transfer of sialic acid from CMP-[^{3}H]sialic acid to asialo-bovine submaxillary mucin

using a disc assay previously detailed [23,24]. The incubation mixture (120 μl) contained: 50 mM Mops (pH 6.5)/5 mM $MnCl_2$/1 mg desialylated bovine submaxillary mucin/20 μM CMP-*N*-acetyl[9-^{3}H]neuraminic acid (0.1 μCi) and enzyme (20–80 μg protein) and incubation was for 60 min at 37°C. At the end of this period duplicate 50-μl aliquots from each of duplicate assays were quantitated. One unit of enzyme activity is the transfer of 1 μmol of sialic acid to acceptor per hour. Adenylate cyclase (EC 4.6.1.1) activity was quantitated by the method of Salomon et al. [25] using [α-^{32}P]ATP. Thiamine pyrophosphatase (EC 2.5.1.3) was assayed by the method of Morré [26]. All marker enzyme activities were determine in duplicate.

Materials

Histone II-A, L-leucyl-β-naphthylamide, DL-isoproterenol-HCl, phenylephrine, dithiothreitol and unlabelled nucleotides were purchased from Sigma Chemical Co., St. Louis, MO. CMP-[9-^{3}H]sialic acid was a product of New England Nuclear Corp., Boston, MA. [*Me*-^{3}H]Thymidine and [α-^{32}P]ATP were obtained from Amersham Corp., Arlington Heights, IL. Bovine submaxillary mucin was a product of Boehringer-Mannheim, Indianapolis, IN.

Results

Plasma membrane purification

In previous work plasma membranes were partially purified from the mouse parotid by homogenization in hypertonic sucrose media and differential centrifugation, with plasma membranes being recovered in the $3 \cdot 10^6$ $g \cdot$ min sediment [16]. The conditions for optimal recovery and purification by this procedure have been re-examined. It is essential that Ca^{2+} is present in the homogenization buffer and in its absence there is a 3–5-fold drop in the specific activity of plasma membrane enzyme markers in the $3 \cdot 10^6$ $g \cdot$ min fraction. However this Ca^{2+} concentration can be lowered to 0.1 mM without altering the sedimentation pattern. The best results are obtained with a teflon/glass homogenizer with a clearance of approximately 0.13 cm and exerting a shear force that breaks 85–90% of the acinar cells. Further purification of the resulting plasma membrane preparation has been achieved by isopycnic centrifugation upon a discontinuous sucrose gradient. Initially the crude plasma membranes were subjected to sucrose gradient centrifugation on a multistep gradient with densities between 1.12 and 1.18 (not shown). Analysis of the distribution of enzyme markers led to the selection of a 3-step gradient of 0.32 M, (*d* 1.04), 1.11 M (*d* 1.14) and 1.31 M (*d* 1.17) sucrose. Two fractions of highly purified membranes are obtained at the interfaces between the 0.32 M and 1.11 M and the 1.11 M and 1.31 M sucrose layers which are called the light and heavy plasma membrane fractions, respectively. The 50000 and 150000 $g \cdot$ min sediments were also subject to sucrose gradient centrifugation but there was little further purification of plasma membrane markers. The fractions have been characterised by using enzyme markers, chemical composition and morphological criteria.

Enzyme markers

Five enzymes were used as markers of the plasma membrane on the basis of their localization to this structure in a wide variety of other tissues: phosphodiesterase, leucylaminopeptidase, 5′-nucleotidase, adenylate cyclase and K^+-activated alkaline phosphatase [16,27]. These enzymes show 26–32-fold and 20–23-fold increases in specific activity compared to the original homogenate in the light and heavy fractions, respectively (Table I). When recovery of the plasma membranes is expressed as a percentage of the total enzyme marker activity recovered, then 27% of the original plasma membrane is estimated to have been found in the light and heavy fractions. Enzyme markers for the microsomes (glucose-6-phosphatase), and mitochondria (succinate dehydrogenase) are extremely low in the purified membrane fractions. Thiamine pyrophosphatase and acid phosphatase were used as markers for the Golgi cisternae and cytosolic vacuoles, respectively, as they have been localized histochemically to these structures in the rat parotid [28]. Approximately 0.5% of the total recovered activity of these enzymes is found in the purified plasma membrane fractions. Sialyltransferase is also generally thought of as an enzyme located in the Golgi apparatus. However 4.8% of its re-

TABLE I

DISTRIBUTION OF ENZYMES IN SUBCELLULAR FRACTIONS ISOLATED FROM MOUSE PAROTID GLANDS

Glands were homogenized and fractionated and enzyme activities assayed as described under Materials and Methods. The enzymes assayed were: I, phosphodiesterase; II, leucine aminopeptidase; III, 5′-nucleotidase; IV, adenylate cyclase; V, alkaline phosphatase; VI, glucose-6-phosphatase; VII, succinate dehydrogenase; VIII, acid phosphatase; IX, thiamine pyrophosphatase; X, sialyltransferase. Activity is expressed relative to the homogenate taken as unity. Activity below the limits of detection is indicated as n.d. Recovery is expressed as the % of homogenate activity recovered in the fraction. Results are the average of two experiments.

Fraction	Protein (%)	Enzyme (relative specific activity)									
		I	II	III	IV	V	VI	VII	VIII	IX	X
6500 $g \cdot min$	25.7	0.91	0.41	0.35	0.64	0.58	3.5	2.7	0.25	1.1	1.8
50000 $g \cdot min$	5.7	3.0	2.0	3.1	2.3	2.5	4.3	4.9	2.2	2.7	2.5
150000 $g \cdot min$	1.4	9.5	7.4	6.4	6.8	7.2	4.3	8.7	3.7	3.1	3.6
$3 \cdot 10^6$ $g \cdot min$	3.0	11.7	14.4	12.1	13.2	12.8	0.17	0.42	0.25	0.39	4.3
Supernate	59.0	0.62	0.87	0.32	n.d	0.73	0.17	n.d	n.d	0.50	0.09
Recovery (%)	94.8	125.5	126.8	90.9	78.7	111.8	131.0	110.8	26.2	78.7	84.4
Plasma membranes											
Light	0.66	26.3	30.3	29.5	32.4	27.4	n.d.	n.d.	0.12	0.42	2.52
Heavy	0.40	19.8	22.9	21.8	23.1	20.8	0.08	n.d	0.10	0.30	5.90
Recovery (%)		25.3	29.2	28.2	30.6	26.4	< 0.1	< 0.1	0.12	0.40	4.0

covered activity is found in the light and heavy plasma membrane fractions suggesting that there is ecto-sialyltransferase activity in the parotid. Electron microscopy of the light and heavy plasma membrane fractions [16] provided confirmation that the contamination of the plasma membranes with Golgi cisternae was minimal (< 2%). Furthermore there were only a very few free ribosomes present and essentially no rough endoplasmic reticulum or mitochondria (not shown).

Chemical composition

The chemical composition of the plasma membrane fractions is shown in Table II. All preparations contain small amounts of RNA. Extraction of this nucleic acid and electrophoresis upon SDS-polyacrylamide gels indicates a heterogeneous population of molecules with no significant peaks of 18 S and 28 S ribosomal RNA (not shown). Since ribonuclease inhibitors are not employed much of this heterogeneity could be artifactual but the low amount of rRNA is in agreement with the electron microscopy and enzyme markers which indicate few ribosomes or rough endoplasmic reticulum. A similar content of surface membrane-bound RNA has been described for L cells [29]. The preparations contain the high cholesterol and sialic acid content and cholesterol: phospholipid molar ratio that are characteristic of surface membranes [30,31]. There was a small but reproducibly higher level of sialic acid in the light fraction. As might be expected from its lower density the light fraction had higher proportions of lipid and carbohydrate.

TABLE II

CHEMICAL COMPOSITION OF PURIFIED MOUSE PAROTID PLASMA MEMBRANES

Plasma membrane fractions were prepared from 80 mice. Aliquots from these preparations were used for all determinations. Values are the average of three experiments.

Component	Plasma membrane fraction (μg/mg protein)	
	Light	Heavy
Total lipid	700	628
Phospholipid	424	372
Cholesterol	164	136
Cholesterol:phospholipid	0.80 [a]	0.76 *
Carbohydrate	96	68
Hexosamines	23.4	27.2
Sialic acid	28.9	26.1
RNA	12	17
DNA	0.8	1.6

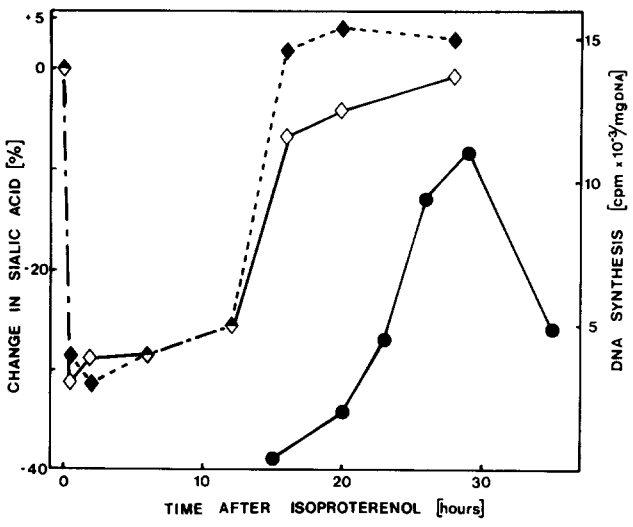
[a] Molar ratio.

Effect of isoproterenol upon the fractionation

The fractionation scheme described above was to be used to study the effect of in vivo administration of isoproterenol upon the properties of isolated plasma membranes. Therefore the effect of this treatment upon the fractionation was investigated. Isoproterenol (0.15 mmol/kg body weight) produces significant changes in the differential centrifugation pattern including a 2-fold increase in the amount of protein recovered in the $3 \cdot 10^6$ $g \cdot$ min sediment with a concomitant fall in enzyme marker specific activities. When this pellet is subjected to sucrose density gradient centrifugation only slightly larger than usual amounts of protein are obtained in the light and heavy plasma membrane fractions but a large amount of protein is now found in the pellet at the bottom of the tube (not shown). Marker enzyme analysis of these light and heavy fractions shows a similar purification of plasma membrane markers to control preparations. Therefore it is possible to directly compare plasma membranes from control and isoproterenol treated mice.

Sialic acid

Evidence has been presented that the stimulation of DNA synthesis in the mouse parotid acinar cell by catecholamine analogs may not involve a coupling of the interaction at the β-adrenergic receptor to stimulation of adenylate cyclase and elevation of cyclic AMP [3]. Isoproterenol administration has been found to lead to a rapid removal of sialic acid from plasma membrane components and it has been suggested that this triggers the events leading to DNA synthesis by an unknown mechanism [14,15]. That work was performed with a partially purified plasma membrane preparation ($3 \cdot 10^6$ $g \cdot$ min). In view of the major change in composition of this fraction when prepared from isoproterenol treated mice it was necessary to repeat the work using the light and heavy plasma



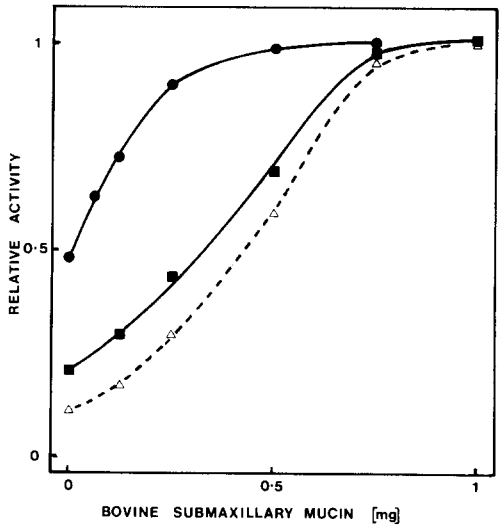


Fig. 1. Time course of changes in parotid plasma membrane sialic acid content in response to isoproterenol. Groups of 50 mice were injected with isoproterenol and after the times indicated light and heavy parotid plasma membranes were prepared. DNA synthesis was measured on groups of four mice. Sialic acid levels were determined in triplicate with < 15% variation between determinations. ◇, Light plasma membranes; ◆, heavy plasma membranes; ●, DNA synthesis.

Fig. 2. Effect of exogenous acceptor upon sialyltransferase activity of purified parotid plasma membranes. Sialyltransferase activity was determined in the presence of varying amounts of desialated bovine submaxillary mucin. ●, $3 \cdot 10^6$ $g \cdot$min pellet; ■, heavy plasma membranes; △, light plasma membranes.

membrane fractions. This confirmed that the loss of sialic acid is from the plasma membrane, and not a contaminating organelle, and the time course of changes in sialic acid content after isoproterenol treatment is shown in Fig. 1 and Table III. Within 30 min there has been a 30% loss of sialic acid, there is no further loss but it remains at this lowered level for at least 6 h. By 12 h a restoration of sialic acid has commenced and between 12 and 16 h there is a rapid return towards control levels,

TABLE III

EFFECT OF ISOPROTERENOL TREATMENT UPON SIALIC ACID CONTENT AND SIALYLTRANSFERASE ACTIVITY OF LIGHT AND HEAVY MOUSE PAROTID PLASMA MEMBRANES

Twenty to fifty mice were used for each experiment. Other conditions were as described in Figs. 2 and 4. Results are the average of three experiments ± S.D.

Treatment	Time after injection (h)	Plasma membrane fraction			
		Sialic acid (μg/mg protein		Sialyltransferase (U/mg protein)	
		Light	Heavy	Light	Heavy
Control	2	28.5 ± 2.7	13.9 ± 1.3	0.26 ± 0.05	0.61 ± 0.05
Isoproterenol	2	20.0 ± 0.8 [a]	9.4 ± 0.8 [a]	0.44 ± 0.04 [a]	0.46 ± 0.07 [b]
Isoproterenol	12	21.1 ± 2.0 [a]	9.9 ± 1.2 [a]	0.53 ± 0.07 [a]	0.93 ± 0.04 [a]

[a] $P < 0.01$.
[b] $P < 0.025$.

although in the light fraction this tails off and normal sialic acid content is not fully restored until 28 h. DNA synthesis commences at about 20 h and peaks at 27 h. Therefore the loss and restoration of sialic acid occurs primarily in the G1 phase of the cell cycle.

Besides a proliferative response to isoproterenol the parotid also responds with secretion. Evidence has previously been presented that conditions causing the secretion of α-amylase but not proliferation do not result in the depletion of plasma membrane sialic acid in partially purified preparations [14]. We have confirmed this observation with purified membranes, using a low dose of isoproterenol or phenylephrine which cause the secretion of more than 50% of parotid α-amylase but no DNA synthesis (not shown). These treatments cause the loss of less than 5% of the plasma membrane sialic acid (Table IV).

A possible role of cell surface sialic acid in the control of cell proliferation is also suggested by the effect of multiple daily isoproterenol injections. After eight injections the parotid has undergone considerable hypertrophy but there is no longer a major cell proliferation or increase in gland size in response to further isoproterenol injection [4]. 24 h after the last isoproterenol injection there has been a more than 2-fold increase in the concentration of membrane sialic acid (Table IV). If a further injection is given and membranes isolated 2 h later the sialic acid content has decreased but the absolute level is still far above normal control values.

Sialyltransferase

The activity of a glycoprotein sialyltransferase in the plasma membrane fractions has been quantitated by measuring the transfer of sialic acid from CMP[^{3}H]sialic acid to desialated bovine submaxillary mucin. The evidence already presented above indicates that the sialyltransferase activity is very unlikely to be due to Golgi contamination. This conclusion is further supported by the fact that whereas overall sialyltransferase activity in the homogenate is markedly stimulated by Triton X-100 the activity in the plasma membrane fraction is strongly inhibited by this detergent [24]. In the absence of an exogenous acceptor there is considerable transfer to endogenous acceptors in the $3 \cdot 10^6\ g \cdot \min$ sediment. Upon further purification much of this endogenous acceptor is lost and there are approximately 5- and 10-fold stimulations of activity in the light and heavy membrane fractions, respectively, upon addition of bovine

TABLE IV

EFFECT OF VARYING CATECHOLAMINE ANALOG INJECTION CONDITIONS UPON PLASMA MEMBRANE SIALIC ACID CONTENT AND SIALYLTRANSFERASE ACTIVITY

Twenty mice were used for each group. Where multiple injections were given they were at 24-h intervals; the number of injections is in brackets, control animals were injected with isotonic saline. Plasma membranes were isolated and the light and heavy fractions combined for the estimation of sialic acid and sialyltransferase activity. Results are the average of three experiments and are expressed with standard deviations.

Treatment		Amount administered (μmol/kg body wt.)	Time after last injection (h)	Sialic acid (μg/mg protein)	Sialyltransferase (U/mg protein)
Control	(1)	–	2	25.1 ± 1.7	0.38 ± 0.04
Isoproterenol	(1)	150	2	15.5 ± 0.9 [a]	0.53 ± 0.05 [b]
Isoproterenol	(1)	1.5	2	23.9 ± 2.4	0.40 ± 0.03
Phenylephrine	(1)	75	2	23.6 ± 1.3	0.40 ± 0.06
Control	(8)	–	24	24.3 ± 3.0	0.39 ± 0.05
Isoproterenol	(8)	150	24	56.9 ± 5.6 [a]	0.77 ± 0.10 [b]
Isoproterenol	(9)	150	2	42.3 ± 5.4 [b]	1.39 ± 0.10 [a]

[a] $P < 0.001$ (versus control).
[b] $P < 0.01$.

submaxillary mucin (Fig. 2). In control parotid the heavy membrane fraction has twice the activity of the light fraction (Fig. 3). This is yet further evidence that the sialyltransferase does not result from Golgi contamination as there are higher levels of Golgi markers in the light fraction.

Isoproternol administration leads to a rapid increase in sialyltransferase activity in the light fraction reaching 2-fold by 12 h. In contrast, there is an initial fall in activity in the heavy fraction followed by a 2-fold rise between 2 and 12 h (Fig. 3 and Table III). These changes in activity do not arise from altered endogenous acceptor levels as a result of sialic acid loss because in neither the light (Fig. 3) nor heavy (not shown) fractions does enzyme activity with endogenous acceptor alter as a percentage of activity with exogenous acceptor. Nor are they the result of changes in nucleotide pyrophosphatase activity as inhibitors of CMP-*N*-acetylneuraminic acid hydrolysis, such as NAD or ATP, do not alter apparent activity and CMP-*N*-acetyl[9-^{3}H]neuraminic acid can be shown to remain intact at the end of the assay by HPLC analysis (not shown). Maximum sialyltransferase activity is thus seen at the same time (12 h) at which maximum restoration of membrane sialic acid is commencing. As sialic acid levels return to close to control values (16 h) this is accompanied by a fall in sialyltransferase activity, indicating that once sialic acid is restored to the membrane the higher activity of sialyltransferase is no longer required. In agreement with the effect upon sialic acid levels, multiple isoproterenol injections lead to elevated sialyltransferase activity to accompany the higher sialic acid level (Table IV).

The properties of the sialyltransferase activity in mixed light and heavy fractions were investigated further. The reaction was linear with up to 80 μg protein for at least 90 min. The pH optimum of the reaction is pH 6.5 (Fig. 4A) but the activity varies greatly with the buffer employed. Mops, imidazole, cacodylate, phosphate and Tris-maleate buffers were all made up at pH 6.5 at 37°C and ionic strength, $I = 0.05$. The relative activity of sialyltransferase in these buffers was 1 : 0.88 : 0.79 : 0.49 : 0.37. The enzyme has a strong requirement for a divalent metal ion with Mn^{2+} being preferred and optimum activity at 5 mM Mn^{2+} (Fig. 4B). Excess Mn^{2+} causes a significant inhibition of activity. The enzyme is sensitive to

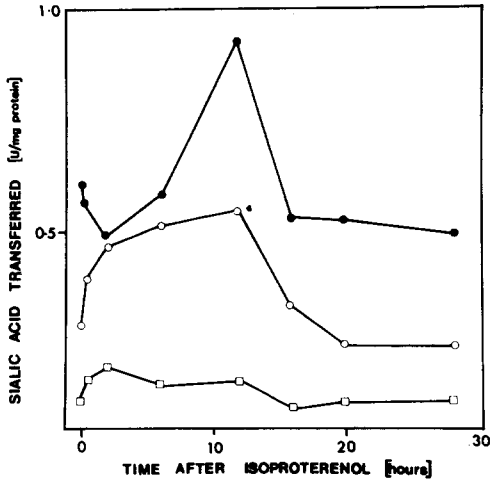


Fig. 3. Time course of changes in sialyltransferase activity of parotid plasma membranes in response to isoproterenol. Light and heavy plasma membranes were prepared as described in Fig. 1 and sialyltransferase activity assayed as described under Materials and Methods with < 15% variation between determinations. ●, Heavy plasma membranes; ○, light plasma membranes; □, light plasma membranes assayed in the absence of bovine submaxillary mucin.

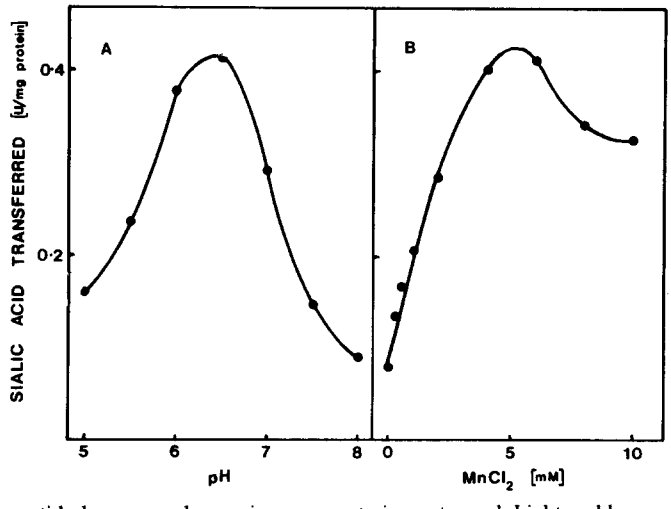


Fig. 4. (A) pH optimum of sialyltransferase activity in mouse parotid plasma membranes. (B) Effect of Mn^{2+} concentration upon mouse parotid plasma membrane sialyltransferase activity. Mixed light and heavy plasma membranes were used. The pH was varied utilizing a mixture of 40 mM Mes and 40 mM Mops and measuring the pH at 37°C in the final incubation mixture.

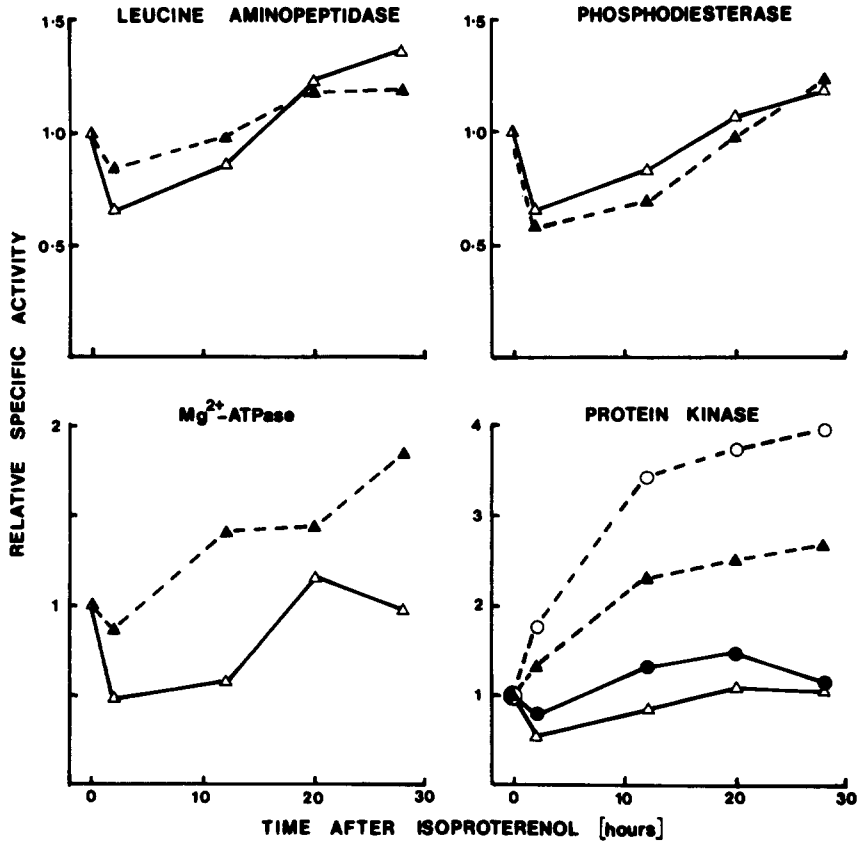


Fig. 5. Enzyme activities of mouse parotid plasma membranes during the pre-replicative period in response to isoproterenol. Light and heavy plasma membranes were prepared as described in Fig. 1, The specific activity of the enzymes was determined as described under Methods and results are expressed relative to control plasma membranes from mice injected with isotonic saline. ○. ▲, Light plasma membranes; ●, △, heavy plasma membranes. Protein kinase activity of the light and heavy plasma membranes, was assayed in the presence of cyclic AMP (○, ●) and in the absence of cyclic AMP (△, ▲).

ionic strength and 100 mM NaCl causes a 40% inhibition of activity. There is no sulfhydryl requirement as dithiothreitol does not activate and sulfhydryl reagents such as *N*-ethylmaleimide and *p*-hydroxymercuribenzoate do not inhibit. Unlike most membrane-bound glycosyltransferases which are activated by Triton X-100 the plasma membrane bound sialyltransferase is strongly inhibited by this detergent [24] and so no detergent is used.

Membrane enzymes

Since the loss of sialic acid will significantly alter the charge of the plasma membrane a number of other membrane-bound enzyme activities were studied to see if a pattern of alteration in activities emerged or whether each individual enzyme was affected in its own particular way. The results for four enzymes are presented in Fig. 5. Leucine aminopeptidase and phosphodiesterase show similar alterations in activity in both light and heavy fractions, and to each other. There is an initial decrease in activity followed by a restoration of activity which quite closely mirrors the loss and restoration of sialic acid (Fig. 1). Protein kinase and Mg^{2+}-ATPase activities also show a similar extent and time course of changes but only in the heavy fraction. In contrast in the light fraction these two enzymes show quite different alterations in activity. Mg^{2+}-ATPase exhibits a small initial decrease in activity followed by a rise in activity to 2-fold control levels by 28 h. Cyclic AMP-dependent protein kinase activity increase

from the earliest time point and by 28 h activity in the presence of cyclic AMP is 4-fold the control level.

Discussion

Highly purified plasma membranes have been obtained from the mouse parotid. From the study of enzyme markers it is concluded that the only significant contamination is with Golgi components. However electron microscopy shows only minimal contamination with Golgi cisternae. There are a number of phosphatase, pyrophosphatase and phosphodiesterase activities in the plasma membrane and it is quite possible that one or more of these enzymes are able to utilize thiamine pyrophosphate and *p*-nitrophenylphosphate to some extent as substrates. Therefore an estimate from enzyme markers of 2% contamination of the light and heavy fractions with Golgi elements from enzyme markers is a maximum possible contamination level.

A number of the markers for the plasma membrane have been found to be confined to specific regions of the cell surface, including adenylate cyclase in the parotid [16]. The fact that five plasma membrane enzymes all show a similar purification would suggest that there is no major loss of any particular area of the cell surface membrane.

The presence of glycosyltransferases at the cell surface is still a matter of some controversy. Originally these enzymes were described as being exclusively located in the Golgi apparatus and smooth endoplasmic reticulum [32]. More recently both sialyltransferase and galactosyltransferase were reported to be found also at the plasma membrane [33,34]. This localization has been challenged but carefully controlled experiments with whole cells [35,36] and the isolation of partially purified plasma membranes enriched in glycosyltransferases [37,38] appear to have established the presence of those enzymes at the cell surface. We have found a marked enrichment of sialyltransferase activity in highly purified mouse parotid plasma membranes which have minimal contamination with Golgi elements and believe that this firmly establishes this enzyme as an intrinsic cell surface component in the parotid. Nonetheless the Golgi is still the major site of sialyltransferase activity as only 10% of the total recovered activity of this enzyme is present in the purified plasma membrane fractions.

The plasma membrane enzyme differs markedly in properties from the Golgi enzymes of other tissues, including porcine submaxillary glands [39,40], in that the Golgi enzymes have no metal ion requirement and are markedly activated by Triton X-100. The plasma membrane enzyme is, however, very similar to an enzyme described in human plasma and lymphocytes which has a strong requirement for Mn^{2+} after dialysis [41] and is strongly inhibited by Triton X-100 [42]. It was suggested that the plasma enzyme was probably derived from lymphocytes [42] and it is interesting therefore that extracellular glycosyltransferases may well come from the plasma membrane of cells. In this regard two lines of evidence suggest that the parotid sialyltransferase is released from the cell upon stimulation with isoproterenol. Firstly there is no increase in intracellular sialyltransferase upon isoproterenol treatment and secondly cannulation of the rat parotid excretory ducts to collect saliva shows that this enzyme is increased more than 50-fold in saliva resulting from isoproterenol treatment as compared to saliva from carbachol treatment [43].

Changes in the activity of cell surface glycosyltransferases with altered proliferation and during the cell cycle have been described but not pattern of changes has emerged. Roth and coworkers [36] have proposed that ectoglycosyltransferases can control proliferation through transglycosylation between cells but there is no direct evidence to support this . In the isoproterenol-stimulated mouse parotid there appears to be a relationship between changes in plasma membrane sialic acid and the activity of sialyltransferase.

Consistent with the hypothesis that isoproterenol induces DNA synthesis in mouse parotid glands by a mechanism not involving cyclic AMP evidence has been presented that sialic acid is rapidly removed from the plasma membrane upon such treatment. Sialic acid is lost only in response to conditions which cause DNA synthesis and is not simply a secretory response. Using a range of catecholamine analogs [3], it has been possible to

correlate the extent of induced DNA synthesis with that of sialic acid loss from the plasma membrane (Durham, J.P., DeValia, V. and Lopez-Solis, R.O. unpublished observations). We have previously shown [44] that if the response to isoproterenol is prevented by the concurrent administration of cycloheximide the tissue is refractory to further isoproterenol for at least 4 h and a full response is not restored until 15 h. This time course of restoration of response closely matches the restoration of cell surface sialic acid seen in Fig. 1. A loss of sialic acid is also seen in regenerating rat liver [45] and a lowering of sialic acid content has been frequently reported for transformed cells [46,47] although increases have also been well documented.

One way in which surface sialic acid residues could regulate cell proliferation is through the action of sialic acid as a calcium-binding molecule [48]. There is considerable evidence that surface bound calcium is the most important in many hormone responses mediated by an influx of Ca^{2+} [49] and 3T3 cells show a large increase in surface bound Ca^{2+} as the cells approach quiescence [50].

While a number of explanations are possible to link this molecular change in the plasma membrane with early cytoplasmic processes also necessary for DNA synthesis we have presented data suggesting that the whole plasma membrane is modified and this is expressed as changes in individual enzyme activities as has also been described in regenerating liver [51,52]. Therefore the regulation of the G_0-G_1 transition and the movement of cells along the proliferative cycle might well be related in a cause effect relationship with the structure of the plasma membrane.

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