The interaction between the adenylate cyclase system and insulin-stimulated glucose transport

Evidence for the importance of both cyclic-AMP-dependent and -independent mechanisms

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The counter-regulatory effect of adenosine, isoprenaline and selected cyclic AMP analogues on insulin-stimulated 3-*O*-methylglucose transport and insulin binding were studied in rat fat-cells. Isoprenaline alone had no consistent effect on glucose transport in the presence of maximally effective insulin concentrations. However, it decreased insulin binding by approx. 20% and increased EC_{50} (concn. giving 50% of maximal stimulation) for insulin from 8 ± 1 to 17 ± 2 μ units/ml. Adenosine deaminase (ADA) alone only exerted a slight effect, whereas isoprenaline and ADA in combination consistently decreased the maximal effect of insulin on glucose transport, decreased insulin binding by approx. 30% and markedly decreased insulin-sensitivity (EC_{50} 61 ± 8 μ units/ml). In cells from pertussis-toxin-treated animals, isoprenaline alone decreased the insulin response by approx. 75%, decreased insulin binding by approx. 45% and caused a marked rightward shift in the dose-response curve for insulin (EC_{50} 103 ± 34 μ units/ml). The importance of cyclic AMP for these effects was evaluated with the analogue *N*⁶-monobutyryl cyclic AMP, which is resistant to hydrolysis by the phosphodiesterase. The importance of phosphodiesterase activation by insulin was studied with 8-bromo cyclic AMP, which is an excellent substrate for this enzyme. *N*⁶-Monobutyryl cyclic AMP, in contrast with 8-bromo cyclic AMP, markedly impaired insulin-sensitivity (EC_{50} approx. 100 μ units/ml). However, the maximal effect of insulin was only slightly attenuated. In conclusion: (1) β -adrenergic stimulation and cyclic AMP markedly alter insulin-sensitivity, but not responsiveness, mainly through post-receptor perturbations; (2) when cyclic AMP is increased phosphodiesterase activation by insulin is a critical step to elicit insulin action; (3) adenosine modulates the insulin-antagonistic effect of β -adrenergic stimulation via N_i (inhibitory nucleotide-binding protein) through both cyclic-AMP-dependent and -independent mechanisms.

INTRODUCTION

Peripheral insulin resistance is rapidly induced *in vivo* by catecholamine infusion (Deibert & DeFronzo, 1980; Sacca *et al.*, 1982; Lager *et al.*, 1986). Detailed studies have shown that catecholamines decrease the response to a maximally effective concentration of insulin and produce a marked rightward shift of the dose-response curve for insulin effects on peripheral glucose uptake (Lager *et al.*, 1986). Owing to the presence of spare receptors in most cells, a decreased maximal effect of insulin (decreased responsiveness) is usually due to a post-receptor perturbation, whereas a rightward shift in the dose-response curve (decreased sensitivity) may be due to either receptor or post-receptor defects (Kono & Barham, 1971).

Studies with isolated fat-cells have shown that, in the absence of adenosine, β -adrenergic stimulation markedly inhibits the maximal effect of insulin on glucose transport. Adenosine accumulating in the incubation medium exerts a potent modulating effect on the ability of β -adrenergic stimulation to inhibit insulin action (Green, 1983; Smith *et al.*, 1984). By using the D-glucose-inhibitable cytochalasin-B-binding technique it was shown that the inhibitory effect of β -adrenergic stimulation was, in part, due to an impairment of the

insulin-stimulated recruitment of glucose-transport units to the plasma membrane, as well as to a markedly decreased intrinsic activity of the glucose transporters (Smith *et al.*, 1984). β -Adrenergic stimulation has also been shown to inhibit the insulin-stimulated redistribution of IGFII-binding sites from the intracellular pool to the plasma membrane (Lönnroth *et al.*, 1985a). Furthermore, insulin binding to fat-cells is rapidly decreased by β -adrenergic stimulation (Lönnroth & Smith, 1983; Pessin *et al.*, 1983; Kirsch *et al.*, 1983; Lönnroth *et al.*, 1985b).

In order to characterize in detail the interaction between the adenylate cyclase system and the activation of glucose transport by insulin, the effect of β -adrenergic stimulation, adenosine, and different cyclic AMP analogues was studied on insulin binding and action. Furthermore, in order to evaluate the importance of the inhibitory nucleotide-binding protein (N_i), experiments were performed in cells exposed to pertussis toxin.

METHODS

Isolated adipocytes

Male Sprague-Dawley rats (150–170 g) were stunned and decapitated, and the epididymal fat-pads excised.

Abbreviations used: ADA, adenosine deaminase; N_i , inhibitory nucleotide-binding protein; EC_{50} , concn. giving 50% of maximal stimulation.

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Fig. 1. Effect of different GTP concentrations on the adenylate cyclase activity in crude membranes of rat adipose cells

The animals were treated with either saline or pertussis toxin 72 h before being killed.

Some of the animals had been injected with purified pertussis toxin (3–5 $\mu\text{g}/\text{animal}$) or, as control, with saline 72 h before decapitation. After mincing, the fat tissue was incubated in plastic scintillation vials with 2 ml of Medium 199 containing 4% (w/v) bovine serum albumin, 5.6 mM-glucose and 1.5 mg of collagenase/ml. The incubations were performed at 37 °C, pH 7.4, with a shaking speed of 140 strokes/min. After 50 min, the cells were liberated by shaking the vials manually, followed by filtration through a nylon mesh. The isolated cells were washed in 4×10 ml of fresh medium and diluted to a lipocrit of about 5–10%.

Insulin binding and lipolysis

Isolated cells were incubated with monocomponent ^{125}I -insulin (0.05–0.12 nM) and different concentrations of unlabelled insulin (0–0.7 μM). After 20 or 60 min incubation at 37 °C, the cells were separated from the incubation medium by centrifugation through silicone oil (Gammeltoft & Gliemann, 1973). Insulin binding was expressed as the cell-associated insulin after subtracting non-specific binding, estimated as the amount of tracer not displaced by the presence of 0.7 μM unlabelled insulin. Lipolysis was measured in 100 μl samples of the incubation medium by measuring the glycerol content enzymically as previously described in detail (Smith *et al.*, 1984). Fat-cell size and number were estimated as described previously (Smith *et al.*, 1972).

3-O-Methylglucose transport

The cells were preincubated in glucose-free medium with 4% (w/v) bovine serum albumin and different concentrations of insulin, isoprenaline, adenosine deaminase, *N*⁶-monobutyl cyclic AMP and 8-bromo cyclic AMP as indicated. After 20 min 3-O-methylglucose transport was measured essentially as described by Whitesell & Gliemann (1979). Briefly, 300 μl of the cell suspension (lipocrit 5–10%) was pulsed with 50 μl of [^{14}C]3-O-methylglucose at a final concentration of 100 μM (0.2 $\mu\text{Ci}/\text{tube}$) for 3–5 s. Each pulse was

terminated by the addition of 2.5 ml of ice-cold phloretin (0.3 mM). After centrifugation through silicone oil, the cells were transferred to scintillation vials and the radioactivity was counted. Trapping of extracellular 3-O-methylglucose was corrected for by adding phloretin before pulsing.

Adenylate cyclase activity

The effect of different GTP concentrations on adenylate cyclase activity was measured in cells from saline- and pertussis-toxin-treated animals. Adenylate cyclase activity was measured in crude membranes with [^{32}P]ATP essentially as described by Hanoune *et al.* (1975).

Materials

Bovine serum albumin (fraction V), collagenase (type I), phloretin, isoprenaline, *N*⁶-monobutyl cyclic AMP and 8-bromo cyclic AMP were from Sigma Chemical Co., St. Louis, MO, U.S.A. Adenosine deaminase was from Boehringer, Mannheim, West Germany. Pig monocomponent insulin was obtained from Novo, Copenhagen, Denmark. [^{14}C]3-O-methylglucose and [^{32}P]ATP were from New England Nuclear, Boston, MA, U.S.A., and monocomponent ^{125}I -insulin was generously given by Dr. Sten Ivarsson, Malmö, Sweden. Pertussis toxin (islet-activating protein) was obtained from List Biological Laboratories, Campbell, CA, U.S.A.

RESULTS

Effectiveness of pertussis-toxin treatment

The effectiveness of the pertussis-toxin treatment to inhibit the N_i action was examined in experiments where the ability of high GTP concentrations to inhibit the adenylate cyclase activity in crude membranes was measured. As shown in Fig. 1, high GTP concentrations inhibited the adenylate cyclase activity in control cells. However, no inhibitory effect was seen in pertussis-toxin-treated cells, thus verifying their loss of N_i function.

Table 1. Effect of isoprenaline and/or ADA on insulin-stimulated rate of 3-*O*-methylglucose transport

The cells were incubated for 20 min with 6.95 nM-insulin with or without the addition of 2 units of ADA/ml and 1 μ M-isoprenaline as indicated before glucose transport was measured. Results are means $\pm$ S.E.M. for six experiments. In Tables 1–3, *P* values for comparisons are shown in parentheses (NS, not significant).

		3- <i>O</i> -Methylglucose transport (amol/s per cell)				
Treatment	Additions to medium . . .	None	Insulin	Insulin + ADA	Insulin + isoprenaline	Insulin + isoprenaline + ADA
NaCl		1.6 ± 0.3	9.7 ± 1.3	8.9 ± 1.3	9.8 ± 1.2	7.4 ± 0.9
		} (< 0.005)	} (< 0.005)		} (< 0.001)	
			} (< 0.025)			
			} (< 0.001)			
Pertussis toxin		0.5 ± 0.1	5.3 ± 0.5	5.0 ± 0.5	2.0 ± 0.3	1.7 ± 0.4
			} (NS)		} (NS)	
			} (< 0.001)			

Table 2. Effect of isoprenaline and ADA on insulin-sensitivity (EC_{50}) for 3-*O*-methylglucose transport in saline- and pertussis-toxin-treated cells

Insulin-sensitivity was measured as EC_{50} values for glucose transport. The animals were injected with saline or pertussis toxin 72 h before being killed. Results are means $\pm$ S.E.M. for six experiments.

Treatment	Additions to medium . . .	Insulin concn. (μ units/ml)		
		Insulin	Isoprenaline	Isoprenaline + ADA
NaCl		8 ± 1	17 ± 2	61 ± 8
		{	{	
			(< 0.025)	
			(< 0.001)	
		(< 0.005)		
Pertussis toxin		17 ± 4	103 ± 24	90 ± 25
		{		{
		(< 0.005)		(NS)

Effect of adenosine deaminase (ADA) and isoprenaline on insulin-stimulated glucose transport

The inhibitory effect of isoprenaline and ADA, alone or in combination, on maximally insulin-stimulated glucose transport was studied in cells from animals injected with either saline or pertussis toxin (Table 1). When added together with insulin, isoprenaline alone had no inhibitory effect, whereas ADA alone caused a slight inhibition. Isoprenaline and ADA together consistently inhibited the insulin effect (20–40%). Cells from pertussis-toxin-treated animals showed a lower basal transport activity and a decreased maximum response to insulin (by approx. 50%). However, the effect of insulin, expressed as relative increment in glucose transport, was unchanged. In these cells isoprenaline alone caused a marked inhibition of insulin action (approx. 70%) and no further effect was seen by the simultaneous addition of ADA (Table 1).

Isoprenaline alone induced a 2-fold rightward shift of the dose-response curve for insulin (Table 2). ADA alone

exerted a similar effect (results not shown). A further decrease in the sensitivity to insulin was seen with the combination of isoprenaline and ADA (EC_{50} approx. 61 μ units/ml). Cells from pertussis-toxin-treated animals showed an insulin-sensitivity similar to that of untreated cells in the presence of ADA (EC_{50} approx. 17 μ units/ml). Isoprenaline alone induced a marked rightward shift in the dose-response curve for insulin (EC_{50} approx. 103 μ units/ml), with no additional effect produced by simultaneous addition of ADA (Table 2).

Insulin binding

Cell-associated 125 I-insulin measured under steady-state conditions after 60 min incubation with isoprenaline alone was decreased by approx. 30%. ADA alone was without effect, whereas the combination of isoproterenol and ADA decreased insulin binding by approx. 50%. Insulin binding in the absence of isoprenaline and ADA was unchanged in pertussis-toxin-treated cells. However, these cells were markedly sensitive to isoprenaline alone;

Table 3. Effect of isoprenaline and/or ADA on ^{125}I -insulin binding

The cells were incubated for 20 min with or without 2 units of ADA/ml and 1 μM -isoprenaline as indicated. Specific binding of 0.05–0.12 nmM - ^{125}I -insulin was then determined. Results are means $\pm$ S.E.M. for four experiments.

Treatment	Additions to medium . . .	^{125}I -insulin bound (%)			
		None	ADA	Isoprenaline	Isoprenaline + ADA
NaCl		5.9 $\pm$ 0.1	5.8 $\pm$ 0.3	4.9 $\pm$ 0.2	3.7 $\pm$ 0.6
		(NS) (< 0.05) (NS) (< 0.005)			
Pertussis toxin		4.9 $\pm$ 0.4	4.5 $\pm$ 0.5	2.5 $\pm$ 0.4	2.1 $\pm$ 0.4
		(NS) (< 0.02) (NS) (< 0.005)			

a 50–60% inhibition of insulin binding was seen, with no additional effect of ADA (results not shown).

In order to obtain data under the same conditions as for the glucose-transport studies, cell-associated ^{125}I -insulin was measured after 20 min incubation with the different agents. Under these conditions, 1 μM -isoprenaline alone decreased insulin binding by approx. 20% and, in combination with ADA, by approx. 35% (Table 3). Isoprenaline alone decreased insulin binding even further (by approx. 45%) in pertussis-toxin-treated cells, but ADA had no additional effect (Table 3). The decrease in binding under these conditions was due to a decrease in the apparent number of receptors (Fig. 2).

Sensitivity to isoprenaline

The dose-response curve for the inhibitory effect of isoprenaline on insulin binding and insulin-stimulated glucose transport is shown in Fig. 3. In control cells half-maximal inhibition of both insulin binding (Fig. 3a) and glucose transport (Fig. 3b) was seen at approx. 0.5 μM -isoprenaline. The dose-response curve for isoprenaline was shifted to the left in the presence of ADA (half-maximal effect at approx. 0.05 μM). Pertussis-toxin-treated cells showed a further enhancement of their sensitivity to isoprenaline, irrespective of whether ADA was present or not (half-maximal effect at approx. 0.02 μM) (Figs. 3a and 3b).

Effect of different cyclic AMP analogues

In order to elucidate the importance of cyclic AMP for the effects exerted by isoprenaline and ADA on both insulin binding and glucose transport, two different cyclic AMP analogues, N^6 -monobutyl cyclic AMP and 8-bromo cyclic AMP, were used. The former analogue is highly resistant to hydrolysis by phosphodiesterase, whereas the latter is an excellent substrate (Beebe *et al.*, 1984). Comparing the inhibitory effect of these analogues on insulin action gives information about the potential cyclic-AMP-dependence of the effects elicited by β -adrenergic stimulation. Furthermore, the importance of phosphodiesterase activation for the metabolic effect of insulin can also be elucidated. The relevance of this experimental model is further supported by the obser-

vation that the elevated intracellular cyclic AMP concentrations and activation of cyclic-AMP-dependent protein kinase effectively decrease the endogenous production of cyclic AMP (Corbin *et al.*, 1985). As shown in Table 4, the two analogues were equipotent in stimulating lipolysis in rat fat-cells in the absence of insulin.

N^6 -Monobutyl cyclic AMP produced a marked shift in the dose-response curve for insulin-stimulated glucose transport (EC_{50} approx. 100 $\mu\text{units/ml}$), whereas it only exerted a small inhibitory effect on the maximal insulin response (Fig. 4a). In contrast, the hydrolysable analogue 8-bromo cyclic AMP had only a marginal antagonistic effect on insulin-sensitivity and did not alter

Fig. 2. Scatchard plot of specific ^{125}I -insulin binding to cells from saline-treated animals after 20 min incubation without (●) or with 1 μM -isoprenaline and 2 units of ADA/ml (○)

Fig. 3. Isoprenaline inhibition of insulin binding (a) and 3-O-methylglucose transport (b)

(a) Dose-response curve for the inhibitory effect of isoprenaline on insulin binding. The cells were incubated with different concentrations of isoprenaline and 0.05–0.12 nM-¹²⁵I-insulin, and the results are expressed relative to the maximal inhibition. Cells from control animals were incubated for 20 min with isoprenaline alone (■) or with 2 units of ADA/ml (●). Pertussis-toxin-exposed cells were incubated with different concentrations of isoprenaline (○). (b) Inhibitory effect of different concentrations of isoprenaline on insulin-stimulated [¹⁴C]3-O-methylglucose transport in control cells in the presence of 2 units of ADA/ml (●). Inhibition by isoprenaline of insulin-stimulated glucose transport in cells exposed to pertussis toxin is shown for comparison (○). Insulin was present at 6.95 nM.

the responsiveness (Fig. 4a). Evaluation of the dose-response curve for the ability of insulin to overcome the inhibition exerted by *N*⁶-monobutyl cyclic AMP showed that the half-maximal effect was also exerted at approx. 100 μunits/ml (Fig. 4b). The inhibitory effect of *N*⁶-monobutyl cyclic AMP and 8-bromo cyclic AMP on the dose-response curve for insulin action on glucose transport was similar in pertussis-toxin-treated cells and in control cells (results not shown). Under both conditions, *N*⁶-monobutyl cyclic AMP produced approx. 10–15-fold increase in the EC₅₀ values, whereas 8-bromo cyclic AMP was essentially without effect in the presence of insulin. Insulin binding was decreased by approx. 30–40% by *N*⁶-monobutyl cyclic AMP, but no clear effect was seen with 8-bromo cyclic AMP (results not shown). The inhibitory effect of *N*⁶-monobutyl cyclic AMP on both insulin binding and insulin-stimulated transport showed similar dose-response relationships; half-maximal effect was obtained at approx. 2 mM.

Table 4. Glycerol release in the presence of different concentrations of *N*⁶-monobutyl cyclic AMP (⁶NcAMP) and 8-bromo cyclic AMP (8-BrcAMP)

The incubations were performed for 20 min with the indicated cyclic AMP analogues. The results are the means of a typical experiment repeated three times and represent the increase over basal release.

Concn. (mM)	Addition to medium . . .	Glycerol release (nmol/10 ⁵ cells)	
		⁶ NcAMP	8-BrcAMP
0.3		7	6
0.5		17	17
1.0		40	63
2.0		158	139
3.0		244	183
4.0		223	197

Fig. 4. (a) Rate of glucose transport at different insulin concentrations without (●) or with 4 mM-8-bromo cyclic AMP (Δ) or 4 mM-*N*⁶-monobutyl cyclic AMP (○); (b) ability of different insulin concentrations to overcome the inhibitory effect of 4 mM-*N*⁶-monobutyl cyclic AMP

The data in (b) are based on the results shown in (a).

DISCUSSION

This study confirms previous findings (Taylor *et al.*, 1976; Green, 1983; Kirsch *et al.*, 1983; Smith *et al.*, 1984) that, in the absence of adenosine from the incubation medium, isoprenaline inhibits the stimulatory effect of a maximal insulin concentration on glucose transport. In addition, it is shown for the first time that isoprenaline and ADA together produce a marked rightward shift in the dose-response curve for insulin. This was associated with a gradual decrease in insulin binding. However, when insulin binding was studied under the same conditions as for glucose transport, i.e. after 20 min incubation, the decrease in insulin binding induced by isoprenaline and ADA was only 20–30%. Thus it is clear that the main reason for the marked decrease in insulin-sensitivity seen with these agents is due to post-receptor perturbations. It has been shown that β -adrenergic stimulation and cyclic AMP decrease the insulin-stimulated phosphorylation of the insulin-receptor tyrosine kinase (Häring *et al.*, 1986; Stadtmauer & Rosen, 1986). It is currently unclear, however, whether this finding may explain the marked insulin-resistance in the cells exposed to ADA and isoprenaline. The additional decrease in insulin-sensitivity seen in cells from pertussis-toxin-treated animals with isoprenaline is probably in part due to the more marked decrease in insulin binding (Table 3).

Pertussis toxin leads to an irreversible modification of the inhibitory nucleotide-binding protein (N_i) (Murayama & Ui, 1983). The fact that N_i was modified by pertussis toxin under the conditions currently used was clearly shown by the loss of inhibitory effect of high GTP concentrations on adenylate cyclase activity, as well as by the lack of effect of ADA in these cells. It is well recognized that adenosine exerts its action via N_i (Londos *et al.*, 1980). However, the fact that adipocytes also contain the pertussis-toxin substrate N_o warrants some caution in attributing all the effects of the toxin to its action on N_i (Neer *et al.*, 1984). Although basal and insulin-stimulated rates of glucose transport were lower in pertussis-toxin-treated cells, the incremental effect of insulin was unchanged. This latter finding would preclude cell damage or other non-specific effects of pertussis toxin as a cause for the lower rates of glucose transport. Moreover, the data strongly support the concept that N_i does not mediate insulin action under normal conditions (Moreno *et al.*, 1983).

The inhibitory effect of isoprenaline and the important modulating effect exerted by N_i on both insulin binding and insulin-stimulated glucose transport strongly suggest that these effects are cyclic-AMP-dependent. The marked shift in the dose-response curve for insulin in the presence of either isoprenaline and ADA or N^6 -monobutyl cyclic AMP supports this possibility. However, also non-cyclic-AMP-mediated effects of adenosine and N_i on glucose transport seem to be involved, since isoprenaline and ADA consistently decreased maximally insulin-stimulated glucose transport, whereas N^6 -monobutyl cyclic AMP was essentially without effect on this parameter. A marked inhibitory effect of isoprenaline alone was seen in pertussis-toxin-treated cells. Taken together, these findings indicate that the decreased insulin-responsiveness seen with isoprenaline and ADA in control cells or with isoprenaline alone in pertussis-toxin-treated cells is, in

contrast with the effect on insulin-sensitivity, largely attributable to a cyclic-AMP-independent mechanism elicited by withdrawal of adenosine and N_i .

The importance of non-cyclic-AMP-mediated effects for the insulin-antagonistic action of isoprenaline and ADA in rat adipocytes has been further substantiated by Simpson *et al.* (1985), who reported that the inhibition was overcome by adenosine analogues without any concomitant decrease in cyclic-AMP-dependent protein kinase activity. However, the interaction between N_i stimulation and insulin is complex, as adenosine analogues binding to the R-type receptors restore the ability of insulin to activate the phosphodiesterase, even in the presence of counter-regulatory hormones (Elks *et al.*, 1983; Wallace *et al.*, 1984; Heyworth *et al.*, 1984, 1986). It is not clear whether such an effect is associated with a lower protein kinase activity. Furthermore, the ability of insulin to overcome the antagonistic effect of 8-bromo cyclic AMP is maintained in pertussis-toxin-treated cells (results not shown).

The importance of phosphodiesterase activation for the ability of insulin to stimulate glucose transport during β -adrenergic stimulation was evaluated by comparing the insulin-antagonistic effects of N^6 -monobutyl cyclic AMP and 8-bromo cyclic AMP. The former analogue is resistant to hydrolysis by phosphodiesterase, but the latter is an excellent substrate (Beebe *et al.*, 1984). The results of those experiments clearly showed that phosphodiesterase activation is necessary to elicit insulin action when cyclic AMP concentrations are elevated. Moreover, the similar EC_{50} value for the effect of insulin to overcome the inhibitory action of the cyclic AMP analogue and to stimulate glucose transport under conditions when the cellular cyclic AMP concentrations are high excludes important additional effects of insulin on cyclic AMP generation, such as inhibition of adenylate cyclase activity. The importance of the phosphodiesterase for insulin action is in agreement with the finding that phosphodiesterase activation is necessary for the hormone to elicit its anti-lipolytic effect (Beebe *et al.*, 1985).

In conclusion, β -adrenergic stimulation and high cyclic AMP concentrations markedly decrease insulin-sensitivity through both receptor and post-receptor perturbations. However, insulin-responsiveness is influenced to a much smaller extent by cyclic AMP. The ability of insulin to modulate the cyclic AMP concentrations by activating the phosphodiesterase is a critical step for the hormone to elicit its effects. Furthermore, adenosine and probably other agents coupled to N_i seem to influence glucose transport through a non-cyclic-AMP-associated mechanism.

This study was supported by grants from the Swedish Medical Research Council, Konrad och Helfrid Johanssons fond, Tore Nilssons fond and the Swedish Diabetes Association. J.I.D. was supported by a grant from the Royal Society, London. The excellent technical assistance by Ulla Carlbrand, Christina Goldmark, Aino Johansson and Eva Weiler is gratefully acknowledged. Skilful secretarial aid was given by Helena Carlsten and Gudrun Jonson.

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Received 14 July 1986/22 December 1986; accepted 19 January 1987