Biochemical Pharmacology, Vol. 34, No. 2, pp. 275–279, 1985.
Printed in Great Britain.

0006–2952/85 $3.00 + 0.00

THE DEVELOPMENT OF TOLERANCE TO ANTILIPOLYTIC AGENTS BY ISOLATED RAT ADIPOCYTES

GARRY D. STRATTON, DAVID D. MYLES,* PETER STRONG and IAN F. SKIDMORE
Department of Biochemistry, Glaxo Group Research, Ware, Hertfordshire SG12 0DJ, U.K.

(*Received* 1 *June* 1984; *accepted* 10 *August* 1984)

Abstract—Using an isolated rat epididymal adipocyte system we have studied the development of tolerance to and cross-tolerance between nicotinic acid, 5-methylpyrazole-3-carboxylic acid and pyridyl-3-tetrazole. Preincubating isolated adipocytes with any one of these compounds results in a reduction of the antilipolytic activity of that compound when the cells are exposed to a subsequent challenge dose. Furthermore, preincubation with nicotinic acid, 5-methylpyrazole-3-carboxylic acid or pyridyl-3-tetrazole results in a reduction of the antilipolytic response to challenge with either of the other two compounds. Preincubation of isolated adipocytes with nicotinic acid does not affect the subsequent antilipolytic activity of the PGE_2 analogue, sulprostone. Preincubation with sulprostone does not lead to the development of tolerance to its own antilipolytic actions. The results obtained from these studies suggest that nicotinic acid, 5-methylpyrazole-3-carboxylic acid and pyridyl-3-tetrazole exert their antilipolytic activity via a common biochemical pathway which is distinct from that mediating the antilipolytic activity of prostaglandins. These findings also indicate that the development of tolerance occurs prior to the involvement of adenylate cyclase in lipolysis.

Chronic pretreatment of rats with nicotinic acid, 5-methylpyrazole-3-carboxylic acid or pyridyl-3-tetrazole results in a reduction in the ability of these antilipolytic agents to reduce plasma non-esterified fatty acid concentrations (NEFA) (the development of tolerance) [1]. Chronic pretreatment of rats with either 5-methylpyrazole-3-carboxylic acid or pyridyl-3-tetrazole results in the development of tolerance not only to their own antilipolytic actions but also to those of nicotinic acid [1]. Similarly, pretreatment of rats with nicotinic acid results in the development of tolerance not only to its own antilipolytic action but also to that of 5-methylpyrazole-3-carboxylic acid and pyridyl-3-tetrazole (the development of cross-tolerance) [1]. These results suggest that these three compounds exert their antilipolytic effects through a common receptor or mechanism.

The mechanism by which tolerance develops to certain antilipolytic agents is unknown. However, it has been suggested that the release of corticosteroids induced by 3-methylisoxazole-5-carboxylic acid [2] and 5-methylpyrazole-3-carboxylic acid [3] plays a role in the development of tolerance to these compounds.

Compounds to which tolerance develops can be of limited therapeutic value, therefore it is important to determine whether potential antilipolytic agents demonstrate this phenomenon. In this paper we have investigated the possibility of using isolated rat epididymal adipocytes to study the development of tolerance and have shown that tolerance does develop to the antilipolytic actions of nicotinic acid, 5-methylpyrazole-3-carboxylic acid and pyridyl-3-tetrazole.

* To whom all correspondence should be addressed.

MATERIALS AND METHODS

Animals. The animals used in these studies were Wistar–Sprague–Dawley cross-bred rats from Glaxo Group Research Ltd., Ware, Hertfordshire, U.K. Rats were fed *ad libitum* on standard laboratory rodent diet, BP No. 1 (BP Nutrition, U.K. Ltd., Witham, Essex, U.K.), except for animals pretreated with nicotinic acid for four days, which were starved 24 hr prior to challenge.

Materials. Nicotinic acid was obtained from Sigma Chemical Co. (London, U.K.). Collagenase (Worthington CLS) was obtained through Flow Laboratories (Irvine, U.K.). Bovine serum albumin (fraction V, microbiological grade) was obtained from Armour Pharmaceutical Co. (Eastbourne, U.K.). (This was dialysed as a 4% solution in Krebs–Henseleit ringer bicarbonate buffer against 10 vol. of the same buffer at 4° overnight to remove non-specifically bound fatty acid). 3-isobutyl-1-methylxanthine (IBMX) was obtained from Aldrich Chemical Co. (Gillingham, U.K.). All inorganic reagents used were Analar grade, supplied either by BDH (Poole, U.K.) or Hopkin and Williams (Chadwell Heath, U.K.). 5-methylpyrazole-3-carboxylic acid, pyridyl-3-tetrazole and sulprostone were synthesised by the Chemistry Research Department, Glaxo Group Research Ltd. (Ware, U.K.).

Studies in isolated adipocytes obtained from rats pretreated with nicotinic acid. Two groups of male rats, 180–220 g, were dosed orally twice daily for 4 days with either vehicle (1% carboxymethylcellulose-CMC, 1 ml/kg body weight) or nicotinic acid (250 mg/kg) (pretreatment doses). After an overnight fast each of the pretreatment groups was subdivided into two groups, one of which was orally dosed with vehicle and the other with nicotinic acid

(250 mg/kg) (challenge doses). Blood samples were collected for the measurement of (NEFA) immediately before this final dose and at 1, 2 and 4 hr afterwards. Other experimental details are as described by Myles *et al.* [1]. After the 4 hr blood sample the epididymal fat pads were removed, isolated adipocytes were prepared and incubations carried out with various challenge doses of nicotinic acid as described below.

Tolerance experiments in isolated adipocytes. The epididymal fat pads from 20 male rats (120–150 g) were used for each experiment. Isolated fat cells were prepared essentially according to the method of Rodbell [4]. The fat pads were scissor-minced in a 1 mg/ml solution of collagenase in Krebs–Henseleit original Ringer bicarbonate [5], with half the recommended concentration of calcium plus 4% bovine serum albumin ('albumin buffer'). Following digestion of the fat pads, the suspension of isolated cells and debris was filtered and then washed three times to remove the collagenase. The washed cells were resuspended in albumin buffer to give a cell concentration of 2×10^5–3×10^5 cells/ml. Aliquots (930 μl)) of the cell suspension were incubated with control or test compound solution for 3 hr at 37° under 95%O_2/5%CO_2 in a shaking water bath. After this 'preincubation', the cell suspensions were washed four times and each group resuspended in fresh albumin buffer to a concentration of 6×10^4 cells/ml.

Aliquots (930 μl) of cell suspension were added to 11 ml polystyrene tubes containing 20 μl test compound solution. The cells were incubated for 15 minutes with the test compound (37°, 95%O_2/5%CO_2) and then 50 μl 2 mM 3-isobutyl-1-methylxanthine (IBMX) was added (to a final concentration of 0.1 mM)—the 'challenge'. Incubation of the cells was terminated after a further hr by the addition of 1 ml 5% perchloric acid. The deproteinised solutions were centrifuged at 3°, 1 ml of the supernatant was added to 125 μl saturated $KHCO_3$ solution, vortex-mixed twice and centrifuged again at 3°. Supernatant

Table 1. The development of tolerance to the antilipolytic action of nicotinic acid in adipocytes prepared from rats pretreated with nicotinic acid

(a) Rats were pretreated and challenged with nicotinic acid (250 mg/kg) as indicated in Methods. Control plasma [NEFA] (mean ± S.E.M.) at 0 hr were as follows: CMC-pretreated rats 0.79 ± 0.07 mM and nicotinic acid-pretreated rats 0.97 ± 0.05 mM. Statistical significance was determined by analysis of variance. The % reduction in plasma [NEFA] caused by nicotinic acid was compared in nicotinic acid pretreated rats with that in CMC-pretreated rats at 95% confidence level: *$P < 0.05$

Time after challenge dose (hr)	% Reduction in plasma [NEFA] following nicotinic acid administration	
	CMC-pretreated rats	Nicotinic acid pretreated rats
1	71.4	43.8*
2	63.8	30.6*
4	62.1	17.9*

(b) Adipocytes from nicotinic acid pretreated rats were incubated as described in Methods. The control (IBMX) rates of lipolysis (μmole glycerol released/10^6 cells/hr) were as follows: CMC-pretreated, CMC challenged 3.29 ± 0.64; CMC-pretreated, nicotinic acid challenged 3.57 ± 0.25 and nicotinic acid pretreated, nicotinic acid challenged 3.21 ± 0.39

Incubation conditions	% Inhibition of IBMX-stimulated lipolysis in isolated adipocytes		
	CMC pretreated; CMC challenged control	CMC pretreated; nicotinic acid challenged	Nicotinic acid pretreated; nicotinic acid challenged
IBMX 0.1 mM	0	0	0
IBMX 0.1 mM + nicotinic acid 1 μM	40	3*	1*
IBMX 0.1 mM + nicotinic acid 10 μM	90	14*	6*
IBMX 0.1 mM + nicotinic acid 100 μM	81	16*	10*

(250 μl) was removed for analysis of glycerol by the method of Eggstein and Kreutz [6] as revised by Bucolo and David [7] using a Calbiochem glycerol assay kit in a Centrifichem 400 centrifugal analyser.

Glycerol release by isolated adipocytes was then taken as an index of lipolysis stimulated by 0.1 mM IBMX in the presence and absence of test compounds. The rate of lipolysis was expressed as μmol glycerol released/10^6 cells/hr. Background lipolytic rates (typically less than 10% of the control, stimulated rates) were subtracted from all other values. The IBMX stimulated rates of lipolysis were not affected by preincubation with any of the compounds. All values were determined in quadruplicate for each experiment unless otherwise stated. All compounds were dissolved in water, except sulprostone which was dissolved in 3% ethanol in 0.9% sodium chloride solution.

Statistical analysis. The results were assessed by analysing the antilipolytic activity of various challenge treatments against IBMX-stimulated lipolysis, (expressed as percentage inhibition with respect to IBMX control), in cells from each pretreatment group. Each group was compared with the control to determine whether the pretreatment affected the subsequent response to antilipolytic compounds. Each point on the test dose–response curve was compared with the corresponding point on the control curve. The statistical analysis was carried out using an analysis of variance test (95% confidence interval, $^*P < 0.05$).

RESULTS

The development of tolerance in isolated rat adipocytes following nicotinic acid pretreatment in vivo

The results shown in Table 1(a) demonstrate the development of tolerance to the ability of nicotinic acid to reduce plasma [NEFA] following pretreatment with this compound. Table 1(b) shows that the antilipolytic effect of 1, 10 or 100 μM nicotinic acid was abolished in adipocytes prepared from rats that either had been chronically dosed with nicotinic acid or had received just a single dose of the compound.

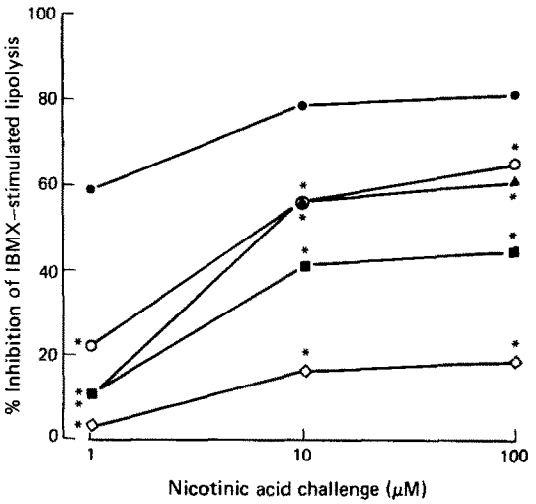

Fig. 1. The effect of preincubation time on the development of tolerance to the antilipolytic effect of nicotinic acid in isolated rat adipocytes. Groups of adipocytes were preincubated with vehicle (●) or 1 mM nicotinic acid for 1 hr (○), 2 hr (▲), 3 hr (■) or 4 hr (◇). After preincubation the cells were challenged with 1, 10 and 100 μM nicotinic acid, as described in Methods. The response of the control cells was the same with all preincubation times and so the mean of the control values has been used in the figure for the purpose of clarity (●). The response of the control cells was compared with that of the nicotinic acid pretreated cells (see 'statistical analysis') Control rates of IBMX-stimulated lipolysis for each pretreatment group were as follows (mean ± S.E.M. expressed as μmole glycerol released/10^6 cells/hr): Control, 4.53 ± 0.74; 1 hr nicotinic acid, 3.13 ± 0.08; 2 hr nicotinic acid, 3.52 ± 0.06; 3 hr nicotinic acid, 6.50 ± 0.01; 4 hr nicotinic acid 4.83 ± 0.04. All determinations were carried out in triplicate. $^*P < 0.05$.

Table 2. The effect of preincubation concentration on the development of tolerance to the antilipolytic effect of nicotinic acid in isolated rat adipocytes. Groups of adipocytes were preincubated for 3 hr as indicated in the table. Control rates of IBMX-stimulated lipolysis for each pretreatment group were as follows (mean ± S.E.M. expressed as μmole glycerol released/10^6 cells/hr): control, 2.10 ± 0.16 (N = 8); 1 μM nicotinic acid, 2.14 ± 0.29 (N = 4); 10 μM nicotinic acid, 2.46 ± 0.35 (N = 4); 100 μM nicotinic acid, 2.20 ± 0.29 (N = 4) 1 mM nicotinic acid 2.02 ± 0.26 (N = 4); $^*P < 0.05$

	% Inhibition of IBMX-stimulated lipolysis in isolated adipocytes following preincubation with nicotinic acid at:				
Incubation conditions	0 μM	1 μM	10 μM	100 μM	1 mM
IBMX 0.1 mM	0	0	0	0	0
IBMX 0.1 mM + nicotinic acid 0.1 μM	7	9	1	−1*	−4*
IBMX 0.1 mM + nicotinic acid 1 μM	39	23*	14*	11*	8*
IBMX 0.1 mM + nicotinic acid 10 μM	64	63	31*	21*	27*
IBMX 0.1 mM + nicotinic acid 100 μM	68	60	33*	34*	22*

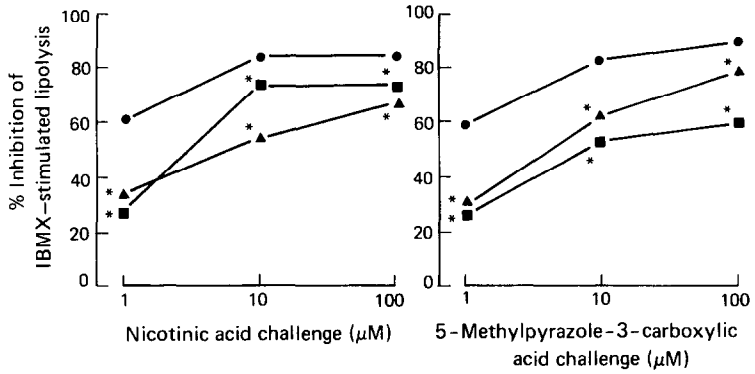

Fig. 2. The development of cross-tolerance to the antilipolytic effects of nicotinic acid and 5-methylpyrazole-3-carboxylic acid in isolated rat adipocytes. Groups of adipocytes were preincubated with control (●), 1 mM nicotinic acid (▲) or 1 mM 5-methylpyrazole-3-carboxylic acid (■) for 3 hr. Control rates of IBMX-stimulated lipolysis for each pretreatment group were as follows (mean ± S.E.M. expressed as μmole glycerol released/10^6 cells/hr): control, 2.60 ± 0.28 (n = 6); 1 mM nicotinic acid, 2.37 ± 0.33 (N = 3); 1 mM 5-methylpyrazole-3-carboxylic acid, 2.71 ± 0.42 (N = 2). *P < 0.05.

The development of tolerance to nicotinic acid in isolated rat adipocytes

The effect of different preincubation times on the development of tolerance to nicotinic acid in the subsequent challenge incubation is shown in Fig. 1. Tolerance to challenge doses of nicotinic acid became more pronounced as the preincubation period with 1 mM nicotinic acid was increased. In all subsequent experiments, a 3 hr preincubation time was used.

The effects of different preincubation concentrations of nicotinic acid on the development of tolerance to subsequent challenge doses are illustrated in Table 2. Preincubation with 1 μM nicotinic acid had no effect on the subsequent antilipolytic activity of challenge doses, whereas preincubation with 10 μM, 100 μM and 1 mM all resulted in the development of the same degree of tolerance.

The development of cross tolerance between nicotinic acid, 5-methylpyrazole-3-carboxylic acid and pyridyl-3-tetrazole

Figure 2 shows that pretreatment of cells with either nicotinic acid or 5-methylpyrazole-3-carboxylic acid resulted in the development of tolerance to the antilipolytic action of both agents. In other experiments (not illustrated) we have also shown the development of tolerance to pyridyl-3-tetrazole and of cross-tolerance between pyridyl-3-tetrazole and nicotinic acid or 5-methylpyrazole-3-carboxylic acid.

Studies with nicotinic acid ans sulprostone

Tolerance did not develop to the antilipolytic action of the PGE_2 analogue, sulprostone, following preincubation with either nicotinic acid or sulprostone (Fig. 3b). The antilipolytic action of nic-

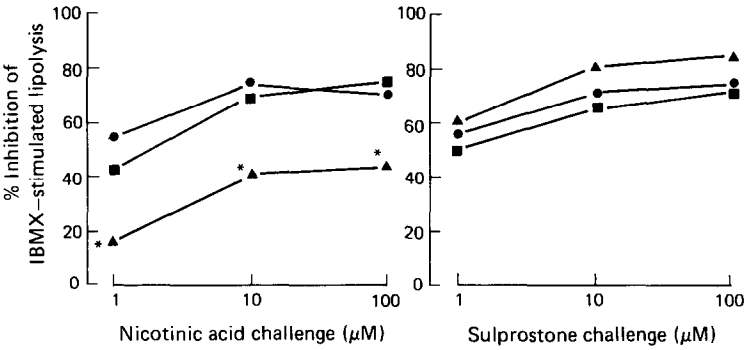

Fig. 3. The effect of preincubating isolated rat adipocytes with nicotinic acid or sulprostone on the subsequent antilipolytic action of nicotinic acid and sulprostone. Groups of adipocytes were preincubated with control (●), 1 mM nicotinic acid (▲) or 10 μM sulprostone (■) for 3 hr. Nicotinic acid was dissolved in water, sulprostone in 3% ethanol in 0.9% sodium chloride solution. Two sets of IBMX controls were obtained in the presence and absence of ethanol/sodium chloride. The results showed that there was no significant difference between the two groups of controls, therefore IBMX control values from both solvent groups were combined. Control rates of IBMX-stimulated lipolysis for each pretreatment group were as follows (mean ± S.E.M. expressed as μmole glycerol released/10^6 cells/hr): 1.96 ± 0.17 (N = 16); 1 mM nicotinic acid, 2.13 ± 0.14 (N = 8); 10 μM sulprostone, 1.50 ± 0.04 (N = 8). *P < 0.05.

otinic acid was reduced following preincubation with nicotinic acid, but was unaltered by preincubation of cells with sulprostone (Fig. 3a).

DISCUSSION

We have shown that tolerance develops to and cross-tolerance develops between the antilipolytic actions of nicotinic acid, 5-methylpyrazole-3-carboxylic acid and pyridyl-3-tetrazole in an isolated adipocyte system following preincubation with any one of these compounds. These results suggest that these structurally related compounds act through a common receptor or mechanism, and that a common mechanism of tolerance induction exists.

Literature reports suggest that the antilipolytic activity of nicotinic acid [8–10] and 5-methylisoxazole-3-carboxylic acid and 5-methylpyrazole-3-carboxylic acid [11] results from an inhibition of adenylate cyclase. This inhibition requires both GTP and sodium ions [10, 11] and is consistent with the concept of the inhibitory activity being mediated via a membrane-bound receptor [12]. The specificity of this inhibitory activity is indicated by our failure to observe cross-tolerance between the PGE_2 analogue, sulprostone, and nicotinic acid. The antilipolytic activity of sulprostone is thought to result from inhibition of adenylate cyclase following interaction of the drug with a prostaglandin receptor on the plasma membrane [13, 14].

Previous workers have reported the development of tolerance to the antilipolytic actions of 3-methylisoxazole-5-carboxylic acid [2] and 5-methylpyrazole-3-carboxylic acid [3] using intact rat models. These reports have implied an involvement of corticosteroid release in the development of tolerance to these compounds. However, data presented here show that tolerance can develop in isolated fat cells, suggesting that a cellular mechanism independent of corticosteroid release exists. Other workers have also recently demonstrated tolerance to the inhibitory effects of antilipolytic agents on adenylate cyclase in members preparations *in vitro*, following treatment of intact hamster adipocytes with 5-methylpyrazole-3-carboxylic acid [11].

Tolerance *in vitro* develops in a manner which is sensitive to both the concentration of the antilipolytic agent and the time the cells are in contact with it. However, the data do not allow us to differentiate between mechanisms involving a down-regulation of the number of receptors such as occurs for insulin [15] or a decrease in the efficiency of coupling between the receptor and adenylate cyclase.

The evidence presented in this paper suggests that the antilipolytic actions of nicotinic acid, 5-methylpyrazole-3-carboxylic acid and pyridyl-3-tetrazole are mediated through a common mechanism, probably involving a specific membrane-bound receptor. This view is supported by the results of Aktories *et al.*, who have recently established that pretreatment of hamster adipocytes with 5-methylpyrazole-3-carboxylic acid does not affect the ability of PGE_1, N^6-phenylisopropyladenosine or adenaline to inhibit adenylate cyclase, and has no effect on the binding of PGE_1 to hamster fat cell membranes [11]. However, the physiological significance of this receptor is not clear at present.

Acknowledgements—The authors thank Carol Kavanagh for typing the manuscript, and David Diss, Mark Johnson and Michele Taylor for technical assistance.

REFERENCES

1. D. D. Myles, G. D. Stratton, P. Strong and I. F. Skidmore, *Biochem. Pharmac.* **34**, 269 (1985).
2. J. N. Pereira and G. F. Holland, *J. Pharmac. exp. Ther.* **157**, 381 (1967).
3. G. C. Gerritsen and W. E. Dulin, *P.S.E.B.M.* **126**, 524 (1967).
4. M. Rodbell, *J. biol. Chem.* **239**, 375 (1964).
5. H. A. Krebs and K. Henseleit, *Hoppe Seyler's Z. Physiol. Chem.* **210**, 33 (1932).
6. M. Eggstein and F. H. Kreutz, *Klin. Wochenschr.* **44**, 262 (1966).
7. G. Bucolo and H. David, *Clin. Chem.* **19**, 476 (1973).
8. I. F. Skidmore, P. S. Schonhofer and D. Kritchevsky, *Pharmacology*, **6**, 330 (1971).
9. Yu-Yan Yeh, *Life Sci.* **18**, 33 (1975).
10. K. Aktories, G. Schultz and K. H. Jakobs, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **312**, 167 (1980).
11. K. Aktories and K. H. Jakobs, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **318**, 241 (1982).
12. K. H. Jakobs, K. Aktories and G. Schultz, *Adv. Cyclic Nucleotide Res.* **14**, 173 (1981).
13. H. Kather, *Prostaglandins, Leukotrienes and Medicine* **8**, 525 (1982).
14. E. Schillinger, G. Prior, A. Speckenbach and S. Wellerschoff, *Prostaglandins* **18**, 293 (1979).
15. G. V. Ronnett, G. Tennekoon, V. P. Knutson and M. D. Lane, *J. biol. Chem.* **258**, 283 (1983).