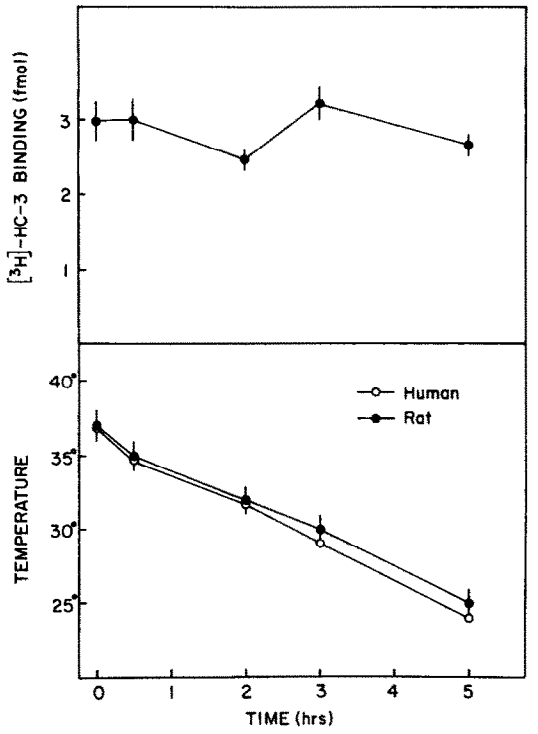

Fig. 2. Effects of slow cooling on specific [^{3}H]HC-3 binding to rat cerebral cortical plasma membranes. Rat brains were placed in non-oxygenated KR at 37° and slowly cooled to room temperature in a water bath incubator. At specified intervals, the temperature of the KR bathing the brains was measured, and then the cerebral cortices were dissected out from these brains to prepare plasma membranes as described in the text. The plasma membranes were then incubated with 2 nM or 10 μM [^{3}H]HC-3, for 30 min at 37° to determine specific binding as described in Methods. Binding values are expressed as mean ± SEM dpm of specific [^{3}H]HC-3 binding. Protein values were consistently between 0.034 and 0.036 mg in these tubes. The human post-mortem brain temperature data were taken from Ref. 7.

Alzheimer's disease and other dementias are associated with profound reductions in cerebral cortical and hippocampal cholinergic function, as measured by changes in choline acetyltransferase activity [9], high-affinity choline uptake [10], presynaptic M_2 muscarinic receptor-binding [11, 12], and ACh levels [9]. We therefore investigated whether [^{3}H]HC-3 binding to high-affinity choline transport sites on plasma membranes may be useful for post-mortem estimation of presynaptic cholinergic activity. Any reduction in the number of transport sites should reflect a loss of cholinergic synthetic capacity [1, 5]. [^{3}H]HC-3 binds to high-affinity choline transport sites on rat brain plasma membranes in a saturable, sodium-dependent, and choline-sensitive manner [2–4]. Published K_D values for specific binding to different brain regions are less than 10 nM, compatible with our results and known IC_{50} values for transport inhibition [2–4].

Using the rat cerebral cortex, we obtained control values from fresh tissue that would be very difficult with humans. Our results suggest that post-mortem clinical analyses of this cerebral cortical [^{3}H]HC-3 binding is feasible, since tissue can slowly cool to room temperature and be frozen as a homogenate without affecting specific binding capacity. Thus it appears that [^{3}H]HC-3 binding may be useful for the post-mortem diagnosis of cortical cholinergic function.

Department of Pharmacology and Experimental Therapeutics RAYMOND D. HAUTAMAKI
EDWIN M. MEYER*
University of Florida School of Medicine
J. Hillis Miller Health Center
Gainesville, FL 32610, U.S.A.

* To whom correspondence should be sent.

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Cromolyn inhibition of protein kinase C activity

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The mode of action of the antiallergic cromolyn drugs on the mast cell is unknown [1–3], and the possibility occurred to us that this might be studied more satisfactorily in the reptilian pigment cell in which the biopharmacological control of melanosome movement is more easily analysed kinetically [4, 5] than release of histamine and other vasoactive agents from mast cell granules. The method used was developed in the lizard *Anolis carolinensis* [6] and the pharmacology upon which our analysis depends is as follows (Fig. 1). In the melanophores of *Anolis carolinensis* as in other lower vertebrates [7, 8] α-MSH induces pigment dispersion mediated by cAMP [9, 10] following interaction with receptors linked to adenylate cyclase [11, 12]. The α_2 adrenoreceptor agonists inhibit the α-MSH response [1^{21}

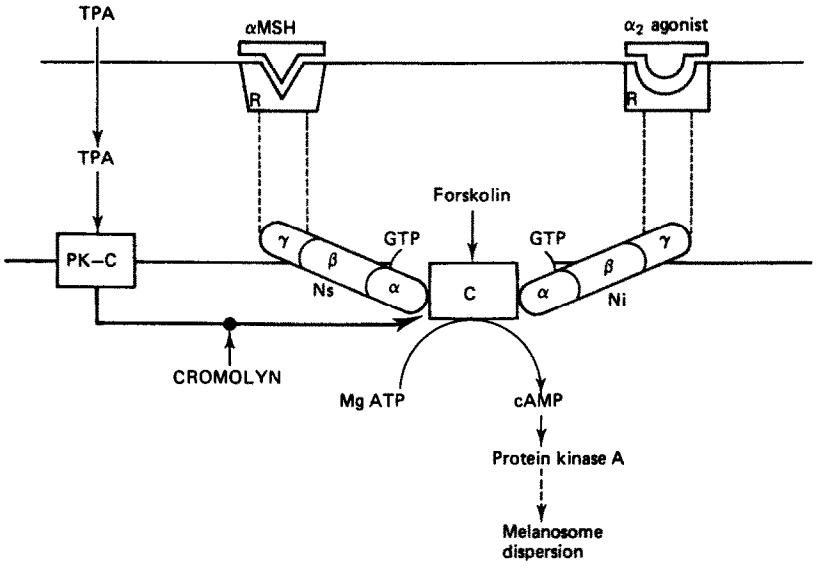

Fig. 1. Cromolyns inhibit TPA-potentiated melanosome dispersion possibly by an inhibition of the increased coupling of the stimulatory nucleotide regulatory subunit (N_s) to the catalytic subunit (C) of adenylate cyclase (see text). N_i = inhibitory nucleotide binding regulatory subunit. PK-C = Protein kinase C. R = receptor proteins. Ca is essential to the system and acts at a variety of points [20, 21] but is omitted from the diagram as it is not involved in cromolyn action.

and it appears that the catalytic moiety (C) of adenylate cyclase in these pigment cells is linked to MSH receptors and α_2 receptors respectively, as in other cell systems [14], via the GTP-binding N_s and N_i nucleotide regulatory subunits (Fig. 1). Stimulation of the calcium-phospholipid dependent protein kinase, protein kinase C, with 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which directly activates the enzyme [15], potentiates the stimulatory effect of α-MSH in *Anolis* in a dose-related manner [16,*] as would be expected from the interaction between the enzyme PK-C and adenylate cyclase. Since the action of both α-MSH and the diterpene forskolin which stimulates the catalytic subunit of adenylate cyclase [17], are potentiated by TPA, whereas the phorbol ester has no effect on the stimulatory response of the melanophore to 8-bromo-cAMP, we have proposed that the interaction between PK-C and adenylate cyclase resides at the level of N_s linkage with C in *Anolis* pigment cells [16] (Fig. 1).

We have used this model of the pigment cells of *Anolis* to examine the effects of two antiallergic drugs, sodium cromoglycate (SCG) and the cromolyn derivative nedocromil sodium (NaNed).

Methods

Assay procedure. The lizards, *Anolis carolinensis*, were obtained from de Natuurvriend, Donkerregard, Utrecht, The Netherlands, and were housed in a terrarium with 14 hr light and 10 hr dark. A temperature gradient of 20–30° was maintained across the terrarium during the light hours while the minimal temperature attained at night was 18°. The lizards were fed with *Tenebrio* larvae and flies and they drank water from the vegetation which was sprayed twice daily.

The rate method of bioassay [6] was used in all investigations. An adult green lizard was decapitated and the trunk skin peeled away from the body wall and floated on the bioassay buffer consisting of 8.3 g NaCl, 0.33 g KCl, 0.21 g Mg $Cl_2.6H_2O$, 0.1 g $NaHCO_3$, 0.16 g $CaCl_2.2H_2O$ and 0.01 g bovine serum albumin per litre of distilled water. The pale ventral and lateral skin and the mid-dorsal skin of the lizard was then cut off and discarded. The remaining skin was then cut into squares of 3 mm × 3 mm. The skin fragments were washed for 1 hr in three changes of the bioassay buffer. α-MSH was diluted with bioassay buffer and a 2-fold dilution series prepared in duplicate in a microtitre plate within the range 3×10^{-9} M to 3×10^{-11} M α-MSH. In all determinations a fixed final volume of buffer (100 μl) was used so that effects were assessed in a dose-related manner. Skin fragments were then incubated in the MSH and the time taken for the skin to achieve 50% darkening was recorded for each solution of the series, the colour being visually assessed over a black background and under constant overhead illumination. This procedure was repeated for each sample dose–response curve. Although the method of assessment was non-blind, subjective bias could not occur since all numerical data was then computer-processed to obtain dose–response and potency values.

The log of the reciprocal of latency against the log dose was plotted for MSH and for each sample. The best fit straight line was calculated using the least squares method and the potency of each dose–response curve computer-calculated relative to that of a α-MSH. Analysis of variance methods were used to calculate the parallelism of the curves, the index of precision of assay, and the 95% fiducial limits of the potency [18]. α-MSH potency is designated as 1.0 throughout. We have used this method of assay to analyse both the effects and site of action of SCG and NaNed on melanosome dispersion. The experiments described in the results and discussion were designed to study the effects of the cromolyns on α-MSH potency, on the TPA-potentiated increase in MSH-induced pigment movement, the forskolin-induced stimulatory response and the effect of modulation of α-MSH and α_2-receptors.

Results and discussion

If our assumption was correct that the mode of action of cromolyns could be analysed through the system outlined (Fig. 1), their overall effect on melanosome movement should be obvious. We therefore looked for an effect of

* A. M. Lucas, S. Shuster & A. J. Thody, in press *J. Endocrinology*.

cromolyns on MSH-stimulated melanophore activity. Standard α-MSH dose-response curves were assessed using duplicate determinations at each of five dose-levels. Within a typical assay the variance of the 10 assay points did not exceed 3×10^{-3} and the index of precision approximated 0.1. For all experiments α-MSH potency was normalised to unity. Nedocromil sodium at 10-fold dilutions ranging from 5 μM to 5 mM showed a dose-related ($r = 0.96$) inhibition of MSH activity, with dose–response curves shifting in parallel ($P > 0.05$) to the right of the standard dose-response curve. The final calculated potencies ranged from 0.36 (±0.068 SEM) at +5 mM NaNed (N = 7) to 0.92 (±0.092 SEM) at +5 μM NaNed (N = 4) (Fig. 2). Similarly SCG at concentrations of 5 μM to 0.5 mM showed a dose related inhibition of MSH potency, although the inhibitory effect was somewhat less with 5 mM SCG (N = 6) (Fig. 2). All inhibitory effects were apparent over the lower dose ranges (<1.5 nM) of α-MSH. Having demonstrated that cromolyns do indeed have an effect on the mechanisms underlying melanosome movement we went on to define their site of action.

Extracellular calcium (Ca^{2+} ext) is a prerequisite for the MSH response but the possibility that SCG or NaNed bound Ca^{2+} in the bioassay buffer was ruled out by the finding of negligible formation of chelated Ca^{2+} salts with the concentration of cromolyns used (Fisons, U.K.). The possibility that the cromolyns act as competitive antagonists of MSH was excluded using Nle^4-D-Phe^7-α-MSH, the analogue of α-MSH which in the assay system used binds preferentially and irreversibly to the MSH receptor [19, 20] and whose stimulatory effects can be modulated by the presence of Ca^{2+}ext [19, 21]. The potency of Nle^4-D-Phe^7-α-MSH was reduced in the presence of 5×10^{-4} M NaNed from 1.0 to 0.70 (±0.145 SEM; N = 3). Using this MSH analogue when skin samples are washed and transferred to fresh medium the pigmentary response is maintained because the analogue is fixed to the receptor unlike α-MSH which is removed by this process [19, 21]. When skin in which the MSH analogue had been fixed to the receptor was exposed to NaNed an inhibitory response was still observed with melanosomes aggregating slowly and transiently in calcium-containing buffer. Confirmation that this was not due to displacement of the MSH analogue from the receptor was obtained by showing that when the skin was then washed and replaced in fresh medium with calcium but without NaNed, the inhibitory effect of the cromolyn disappeared and the pigmentary response to the MSH analogue returned to the level achieved before treatment. Thus the inhibitory effect of the cromolyns is not by competition with the MSH at the receptor site.

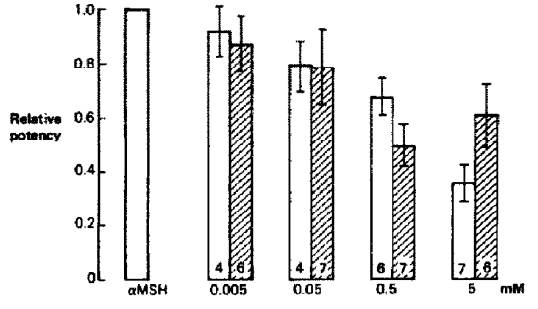

Fig. 2. Increasing concentrations of NaNed (5 μM–5 mM) (open bars) showed a dose-related ($r = 0.96$) inhibition in α-MSH potency (designated as 1.0). SCG (5 μM–5 mM) (hatched bars) also reduced α-MSH potency. Error bars represent ± SEM and number of potency determinations is shown within each histogram block. Each potency determination was calculated from dose–response curves incorporating a minimum of 5 different α-MSH doses, with duplicate estimations at each dose. Only parallel ($P > 0.05$) dose–response curves were compared. This procedure was maintained throughout. SCG and NaNed were obtained from Fisons Pharmaceuticals, Loughborough, U.K., and were freely soluble in the aqueous bioassay buffer. Skin fragments were incubated in each dose of cromolyn for at least 20 min before assay.

Addition of 8-bromo cAMP (1 mM) to cromolyn-inhibited melanophores, restored the pigmentary response and, in addition, the dose–response curve for 8-bromo cAMP (1.56×10^{-4} M–10^{-2} M) remained unaltered by any dose of cromolyn. Thus with 5 mM NaNed the potency of 8 bromo cAMP was 1.017 ± 0.103 (N = 3) and with 5 μM NaNed the potency of 8 bromo cAMP was 1.033 ± 0.127 (N = 3), compared to the standard 8 bromo cAMP potency designated as 1.0. This indicates that the point of inhibitory action of the cromolyns precedes that of cAMP synthesis, and thus excludes mechanisms such as inhibition of intracellular cytoskeletal protein assembly, which would occur distal to cAMP production. In addition, the pigmentary response to forskolin (40 nM–5 μM) was not affected to any great degree by inhibitory doses of cromolyns. Thus in the presence of 5 mM NaNed the potency of forskolin was 0.87 ± 0.15 (N = 3) compared to the standard forskolin potency designated as 1.0. Furthermore, this indicates that the point of action of cromolyns is proximal to the catalytic subunit of AC (Fig. 1).

Thus cromolyn could act either by an α_2-agonistic effect or by inhibition of PK-C. By experimental modulation of the α_2-adrenoreceptor action we have excluded the former. Thus, the α_2-agonist clonidine hydrochloride (5 μM) reduced the TPA-potentiated response of MSH (% inhibition = 89.1%; 95% fiducial limits of 87.7% and 90.4%), and this reduction could not be further depressed by the addition of 2.5 mM NaNed, although this was perhaps not surprising since the α_2-inhibiting effect (90%) was almost maximal. Clonidine (5 μM) also reduced the forskolin response from 100% to 6% and this might suggest that the cromolyns were similarly acting via an α_2-type inhibition. However, the mediation of an α_2 mechanism in the action of cromolyns was conclusively excluded by the finding that the α_2-antagonist yohimbine (5 μM) had no effect on the MSH inhibitory action of nedocromil sodium (5 mM), with the resulting potency having a value of 0.40 (95% fiducial limits of 0.34 and 0.47).

Thus by exclusion, the action of the cromolyns was localised to an inhibition of PK-C. To test this positively we studied the effect of TPA which stimulates PK-C. As previously reported [16], we confirmed that TPA (16 nM) potentiated the effect of MSH (potency range 1.5–2.2; N = 4). This potentiation was greatly reduced by both NaNed and SCG, with dose–response curves shifting in parallel ($P > 0.05$) to the right of the MSH standard dose–response curve. The resulting inhibition of both α-MSH and TPA potentiated α-MSH potencies are shown in Table 1. It can be seen that all doses of cromolyn showed significant depression of the α-MSH + TPA response, over and above the reduction in potency obtained with α-MSH + cromolyn alone.

In summary, we have demonstrated that cromolyns inhibit melanophore movement by inhibition of the action of PK-C thereby preventing the coupling of N_i and C (Fig. 1) and this conclusion can now be further tested by direct measurement. It has been suggested [2, 22] that cromolyn may act to inhibit histamine release from rat peritoneal mast cells by increasing the phosphorylation of a specific 78K mast cell protein which regulates the natural termination of the secretory response. Although the detailed basis of this effect remains obscure it has been implicated to involve the interaction of cromolyn with PK-C [3], and the present results would support this suggestion of a cromolyn–enzyme interaction. Because we have used a cell other than the mast cell and studied both SCG and a

Table 1. The inhibitory effects of NaNed and SCG on α-MSH and TPA-potentiated α-MSH potency

	NaNed				SCG			
	% inhibition of α-MSH potency ± SEM	N	% inhibition of TPA-potentiated α-MSH potency ± SEM	N	% inhibition of α-MSH potency ± SEM	N	% inhibition of TPA-potentiated α-MSH potency ± SEM	N
5 μM	6.2 ± 5.0	4	57.6 ± 4.0	3	12.3 ± 10.2	6	48.0 ± 8.0	3
50 μM	21.0 ± 9.3	4	66.4 ± 3.7	3	21.5 ± 14.6	7	81.0 ± 4.0	3
500 μM	32.2 ± 7.0	6	84.5 ± 3.2	3	50.6 ± 8.0	7	74.5 ± 6.5	3
5 mM	64.5 ± 6.8	7	89.9 ± 1.4	3	39.4 ± 11.9	6	79.5 ± 5.0	3

derivative, the mechanism which we have found is likely to be a general property of cromolyns which we presume underlies their therapeutic effect. Both the principle and the assay therefore provide a new method for development of more potent mast cell "stabilising" agents.

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Department of Dermatology
University of Newcastle upon Tyne, U.K.

ANGELA M. LUCAS
SAM SHUSTER

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β-Lactam antibiotics and transport via the dipeptide carrier system across the intestinal brush-border membrane

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It is well established that the small intestine can transport amino-β-lactam antibiotics with an α-amino group in the side chain such as amoxicillin, cyclacillin, cephalexin, cephradine and cefadroxil, which have very low lipid solubility, via the dipeptide carrier system(s) [1–5].

Until now, α-amino-group-deficient β-lactam antibiotics such as benzylpenicillin and propicillin have been believed to be absorbed by simple diffusion restricted by the brush-border membrane lipid barrier [6–8]. However, it is unclear whether an α-amino group on the side chain of the antibiotic is essential for transport by the dipeptide carrier system(s) or not.

The purpose of this study was to characterize the intestinal transport of cefixime* (FK027) (Fig. 1), a new oral cephalosporin antibiotic, using the intestinal brush-border membrane vesicles (BBMVs), and to elucidate what kind of β-lactam antibiotics have the potential ability to be transported across the brush-border membrane via the dipeptide carrier system(s).

Materials and methods

The β-lactam antibiotics used in this work were supplied as follows: cefixime (CFIX), FK089, ceftizoxime and cefazolin by the Fujisawa Pharmaceutical Co., Osaka, Japan; cyclacillin (ACPC) and propicillin (PPPC) by Takeda Chemical Industries, Osaka; benzylpenicillin (PCG), phenoxymethylpenicillin (PCV) and dicloxacillin (MDIPC) by Meiji Seika Kaisha, Tokyo, Japan; cephradine by the Sankyo Co., Tokyo; D-cephalexin (D-CEX) by Shionogi & Co., Osaka; carbenicillin by the Taito Pfizer Co., Tokyo; and L-cephalexin (L-CEX, custom-made) by the Asahi Chemical Industry Co., Tokyo.

* Abbreviations: cefixime, (6*R*,7*R*)-7-[(Z)-2-(2-amino-4-thiazolyl)-2-(carboxymethoxyimino)-acetamido]-8-oxo-3-vinyl-5-thia-1-azabicyclo(4.2.0)oct-2-ene-2-carboxylicacid; FK089, (6*R*,7*R*)-7-[(Z)-2-(4-thiazolyl)-2-(carboxymethoxyimino)acetamido]-8-oxo-5-thia-1-azabicyclo(4.2.0)oct-2-ene-2-carboxylic acid; and HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.