Effects of the glycine prodrug milacemide (2-N-pentylaminoacetamide) on catecholamine secretion from isolated adrenal medulla chromaffin cells

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1 Milacemide (2-n-pentylaminoacetamide) is a glycine prodrug which readily crosses the blood brain barrier and increases brain glycine and glycineamide. *In vitro* and *in vivo* studies, with numerous tissues, including adrenal chromaffin cells, have clearly shown that the formation of the latter metabolites is exclusively mediated by monoamine oxidase B for which milacemide is a substrate.

2 Milacemide, glycineamide and glycine caused a time- and dose-dependent release of catecholamines from bovine isolated chromaffin cells.

3 Milacemide (10^{-4} M) induced catecholamine release was roughly 30% of that initiated by acetylcholine (10^{-4} M), the natural secretagogue.

4 The combined effects of milacemide (10^{-4} M) and acetylcholine (10^{-4} M) on catecholamine secretion from chromaffin cells is additive, suggesting that milacemide does not act through the normal nicotinic receptor release mechanism.

5 The release of catecholamines from chromaffin cells in response to milacemide (10^{-4} M) was partially inhibited by the selective MAO-B inhibitors (–)-deprenyl (10^{-7} M) and AGN 1135 (10^{-6} M). This indicates that the MAO-B derived metabolites, glycineamide and glycine, contribute to the secretion of catecholamines as does milacemide itself.

6 It is apparent that release of catecholamines by glycine is mediated by its uptake into the cells since [³H]-glycine uptake and catecholamine release showed a highly significant correlation ($r = 0.96$).

Keywords: Milacemide; glycine; monoamine oxidase; adrenal medulla; chromaffin cells; catecholamine

Introduction

Milacemide (2-n-pentylaminoacetamide) is a novel glycine prodrug anti-convulsant (Roba *et al.*, 1986) which has been shown to be readily taken up by the brain and converted to glycineamide and glycine. This conversion is mediated by an N-dealkylating pathway, involving solely monoamine oxidase B (MAO-B) activity in the brain and systemic organs, and includes a secondary transamination of glycineamide to glycine (de Varebeke *et al.*, 1988). Elevation of brain glycine concentration may be the key reaction in the anticonvulsant effect of milacemide, since glycine is an inhibitory amino acid neurotransmitter with anti-convulsant activity (Houtkooper *et al.*, 1986). The secondary amine nature of milacemide and its conversion to glycine specifically via the oxidative reaction of MAO-B (de Varebeke *et al.*, 1988), indicates a central role for the latter enzyme in milacemide anti-convulsant activity, since the MAO-B inhibitor 1-deprenyl prevents the increase in seizure threshold in milacemide-treated rats (Youdim *et al.*, 1988). Milacemide is also thought to increase vigilance in epileptic subjects (Saletu *et al.*, 1986), a feature associated with increased blood levels of catecholamines and their metabolites.

The adrenal medulla chromaffin cell, the main function of which is the storage and secretion of catecholamines, could be useful as a model system to elucidate the mechanism of action of milacemide (Youdim, 1991) in the brain, since the chromaffin cell contains only MAO-B activity (Youdim *et al.*, 1984) and is capable of dealkylating milacemide to glycinamide (de Varebeke *et al.*, 1988; 1989). This cell has also been used as a model for investigating the mechanism of release of catecholamines and other neurotransmitters (Livett, 1984), and has been shown to have both γ -aminobutyric acid (GABA) and benzodiazepine recognition sites (Kataoka *et al.*, 1986; Kuma-

kura *et al.*, 1987) similar to those found in the brain (Bormann & Clapham, 1985; Tauck *et al.*, 1988). These studies suggest the possibility that the adrenal medulla might be a target organ for other amino acid neurotransmitters as well. We have, therefore, investigated the action of milacemide on catecholamine release from bovine isolated chromaffin cells.

Methods

Cell preparation

Bovine adrenal glands were obtained from the local slaughterhouse and processed within 1 h of slaughter. The whole glands were retrogradely washed via the adrenal vein with Krebs-Ringer buffer (pH 7.4) (composition, mM: NaCl 114, KCl 4.7, MgSO₄ · 7H₂O 1.2, CaCl₂ · 2H₂O 2.2, glucose 10, KH₂PO₄ 1.2, NaHCO₃ 25, bovine serum albumin 57% and ascorbic acid 2%) containing HEPES (25 mM). The tissues were then processed as previously described (Hiram *et al.*, 1984). Briefly, the tissue was retrogradely perfused with 0.2% collagenase (Sigma, St. Louis, MO, U.S.A.) in the above buffer solution, the cortex was dissected away from the partially digested medulla and discarded. The medulla was minced and re-introduced to the collagenase solution and incubated, with rapid shaking, at 37°C for 30 min. The resulting cell suspension was washed, filtered through 150 mesh nylon and then resuspended in the Krebs-Ringer buffer (pH 7.4) containing 25 mM HEPES and 0.5% (w/v) bovine serum albumin. The cells were then purified by differential centrifugation on a Percoll gradient (Livett *et al.*, 1987) and then were allowed to recover for 1 h at 37°C.

Incubations

Following the recovery period (1 h), the cells were centrifuged at 300 g, washed twice with warm HEPES-Krebs buffer and

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then resuspended with enough buffer to contain approximately 10^6 cells per ml. One ml of cell suspension was used for each incubation tube, to which additions were made with a total volume not exceeding $20\ \mu\text{l}$. Cells were incubated in the presence or absence of acetylcholine (ACh) at $10^{-4}\ \text{M}$ for control values, and with various concentrations of milacemide, glycine and glycineamide. Standard incubation conditions were 20 min at 30°C , at which maximum secretion of catecholamines is seen with the adrenal medulla chromaffin cells (Hiram *et al.*, 1984).

Catecholamine determination

Catecholamine secretion was measured by determination of the concentration in the medium at the end of the incubation period following separation from the cells by rapid centrifugation at $500\ g$ for 5 min. Total catecholamine was determined after osmotic lysis of the incubated cells in twice distilled water. Catecholamine determination was by a slight modification (Greenberg & Zinder, 1982) of the spectrofluorometric method of von Euler & Folding (1955).

Uptake measurements

[^3H]-glycine (NEN, $49\ \text{Ci mmol}^{-1}$) at a concentration of $10^{-6}\ \text{M}$ was added to the medium. At the end of the incubation period cells were spun down, washed twice with 5 ml buffer to remove adherent counts and then lysed. The radioactivity in the total lysate was counted with Lumax:Toluene (1:2) as the scintillation liquid. The cells were washed thoroughly (twice) to free them from adhering radioactive glycine before lysing them to determine intracellular radioactivity.

Protein measurement

Protein values were determined by a modification of the Bradford method (Bradford, 1976). The cell pellet obtained in the spontaneous release vials was resuspended in 5 ml 0.9% NaCl solution and centrifuged again to recover the washed cells. The cells were then resuspended, lysed in 0.5 ml 0.25 N NaOH and then incubated for 10 min at 37°C . At the end of the incubation, 0.5 ml distilled water was added; $100\ \mu\text{l}$ samples were added to Coomassie reagent and the optical density was determined at 495 nm.

Drugs

Acetylcholine chloride was obtained from Fluka and glycine from Sigma Chemical Co. Milacemide and glycineamide of highest purity were gifts from Searle Co. (Belgium). (–)-Deprenyl was kindly provided by Professor M. Da Prada, Hoffman La Roche (Basel, Switzerland) and AGN 1135 (N-propargyl-aminoindane) by Aspro Nicholas (Australia). [^3H]-glycine ($49\ \text{Ci mmol}^{-1}$) was purchased from NEN. Sodium bicarbonate was obtained from Frutarom. All other materials of the highest purity obtainable, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

The release of catecholamines from bovine isolated adrenal medulla cells by both milacemide and its catabolic products, glycine and glycineamide, is dose-dependent as seen in Figure 1. It is apparent that glycine is more potent than milacemide and this is probably also true for glycineamide, suggesting that perhaps they are the active agents in causing catecholamine release. In Table 1, the effect of milacemide on catecholamine secretion is compared to that of acetylcholine (ACh), the natural secretagogue for the adrenal medulla chromaffin cell. The effect of milacemide is approximately 30% of that exhibited by ACh, when both are used at the concentrations

Figure 1 Dose-response curves for milacemide-, glycineamide- and glycine-induced catecholamine release from isolated adrenal medulla chromaffin cells. The cells were incubated with drugs for 20 min at 30°C : milacemide ($\square$), glycineamide ($\bullet$) and glycine ($\blacksquare$). The results are expressed as a percentage of total initial cellular catecholamines as determined in the zero-time incubation and are the mean from 4 experiments (s.e.mean shown by vertical bars), in each of which measurements were made in triplicate.

which give maximum response ($10^{-4}\ \text{M}$) (Livett & Boksa, 1983). When combined, their effect was additive (Table 1).

(–)-Deprenyl, a specific MAO-B inhibitor, was used in order to block the formation of glycine from milacemide by inhibiting the activity of MAO-B present in chromaffin cells (Youdim *et al.*, 1984). (–)-Deprenyl itself caused some release (30% of milacemide effect alone at a concentration of $10^{-6}\ \text{M}$). When this was subtracted from the effect with milacemide $10^{-4}\ \text{M}$ and deprenyl, a total release similar to that found under basal conditions was observed (Figure 2), suggesting that the effect of milacemide is probably mediated by its breakdown products. Another specific MAO-B inhibitor, AGN 1135 (Kalir *et al.*, 1981), also inhibited the effect of milacemide at $10^{-6}\ \text{M}$ (Figure 2), resulting in catecholamine secretion which was 45% of that seen with milacemide alone.

The time-course of catecholamine release using milacemide ($10^{-4}\ \text{M}$) and glycine ($10^{-6}\ \text{M}$) is shown in Figure 3. Secretion, stimulated by $10^{-6}\ \text{M}$ glycine, peaks at 3 min, while that stimulated by milacemide is significant only after 3 min incubation, reaching peak levels at 5 min (Figure 3). This is again consistent with the proposition that milacemide is metabolized to give the active agent, glycine. The concentrations of glycine and milacemide used in the experiment were those that caused similar levels of secretion (Figure 1).

In order to investigate whether the effect of glycine is due to its uptake into the cell, the uptake of [^3H]-glycine was determined simultaneously with catecholamine release. The time-course of the two effects was very similar (Figure 4). The

Table 1 Stimulation of catecholamine secretion by isolated chromaffin cells in response to milacemide and acetylcholine

Agent	n	Total catecholamine release (nmol mg^{-1} protein)
Control	13	12.5 ± 4.0
Acetylcholine	15	$87.5 \pm 10.0^{**}$
Milacemide	12	$25.3 \pm 7.0^*$
Acetylcholine + milacemide	12	$107.0 \pm 10^{**}$

All drugs were present at a concentration of $100\ \mu\text{M}$. Incubations were for 20 min at 30°C as described in Methods. The results are expressed as means $\pm$ s.e.mean of n experiments, in each of which measurements were made in triplicate.

* $P < 0.05$ (compared with control); ** $P < 0.001$ (compared with control).

Figure 2 The effect of selective monoamine oxidase B (MAO-B) inhibitors on milacemide-induced catecholamine release from isolated adrenal medulla chromaffin cells. The cells were incubated with or without the concentration of MAO-B inhibitors indicated for 20 min at 30°C in addition to milacemide (10^{-4} M). The results are the mean from 4 experiments; s.e.mean shown by vertical bars. The release due to deprenyl alone was subtracted from the total release in the presence of milacemide + deprenyl. C, control (milacemide alone).

correlation coefficient between [3 H]-glycine uptake and catecholamine secretion ($r = 0.96$) is highly significant, using the Pearson correlation, providing strong evidence that intracellular glycine is apparently stimulating release.

Discussion

The present study clearly shows that the glycine prodrug, milacemide, induces a dose- and time-dependent catecholamine release from isolated adrenal chromaffin cells. If milacemide crosses the chromaffin cell membrane as easily as it translocates across the blood-brain barrier (Roba *et al.*, 1986), it is reasonable to assume that the intracellular MAO-B present in the chromaffin cell will cleave it (Youdim *et al.*, 1984), resulting in elevated intracellular glycine concentrations. Youdim *et al.* (1984) found the exclusive presence of MAO-B activity in adrenal medulla chromaffin cells by demonstrating that in frozen and thawed or intact chromaffin cells the MAO-B oxidizes milacemide and other MAO-B substrates such as phenylethylamine with the same efficiency as homogenates from other MAO-B containing tissues. Indeed, our recent (unpublished) studies using intact bovine chromaffin cells have clearly shown that these cells can take up milacemide quite readily and oxidize it using intracellular MAO-B, with a relative K_m of 7.8×10^{-5} M, which is very close to that found with chromaffin cell homogenate MAO-B (K_m , 5.8×10^{-5} M) (de Varebeke *et al.*, 1988). This suggests that uptake is not limiting in the metabolism of milacemide. Furthermore, (-)-deprenyl, rather than clorgyline (MAO-A selective), inhibits milacemide oxidation (de Varebeke *et al.*, 1988). Whether the secretion stimulated by milacemide is a direct effect on the cells or an indirect effect involving intracellular oxidation of milacemide to glycinamide and glycine by monoamine oxidase B (de Varebeke *et al.*, 1988) or a combination of both, is not clear as yet. The inhibitory effect of (-)-deprenyl and AGN 1135 (Kalir *et al.*, 1981), both specific MAO-B inhibitors, on the stimulation of catecholamine secretion by milacemide (Figure 2) is highly suggestive that the milacemide effect is via its oxidation products. The results of the present study also show that glycine is taken up by the adrenal medulla chromaffin cells and that when glycine is incubated with the cells, catecholamine release follows. This

Figure 3 The time course of (a) total catecholamine release and (b) drug-induced secretion from isolated adrenal medulla chromaffin cells in response to milacemide and glycine. The cells were incubated at 30°C for the times indicated with milacemide (10^{-4} M) (○) or glycine (10^{-6} M) (△). The drug-induced secretion of catecholamines was calculated by subtracting the spontaneous release from the amount found in the medium at each time point. The results are the mean from three experiments, each with triplicate observations; s.e.mean shown by vertical bars.

evidence strongly suggests that milacemide is indeed a prodrug delivering glycine to an intracellular site of activity. Milacemide does not seem to act through the normal nicotinic-receptor release mechanism since when administered together with acetylcholine, the natural secretagogue, catecholamine release is enhanced (Table 1). Numerous studies (Livett & Boksa, 1983; Marley, 1988) have shown that maximal stimulation of secretion is reached at 10^{-4} M acetylcholine, while higher concentrations reduce the secretory response. If milacemide were acting through the normal nicotinic receptor pathway, release greater than that seen with 10^{-4} M ACh would not occur. The possibility that milacemide acts by amplifying the cellular response to ACh stimulation cannot, however, be excluded. The ability of milacemide to increase vigilance in epileptic subjects, as demonstrated by Sallet *et al.* (1987), may thus be related to its catecholamine-releasing property, not only in the adrenal medulla system, but perhaps centrally as well.

The direct effect of glycine and glycinamide on catecholamine secretion, and the demonstration that release occurs together with the rise in cytoplasmic glycine, suggests that these metabolites are able to traverse the plasma membrane

Figure 4 Time course of [³H]-glycine uptake (●) and catecholamine release (▲) from adrenal medulla chromaffin cells. The cells were incubated with [³H]-glycine (10^{-6} M) for the times indicated. At the end of this period catecholamine release was determined in the medium after harvesting the cells for determination of [³H]-glycine uptake. The results are the mean from 5 experiments, each with triplicate observations; s.e.mean shown by vertical bars.

and to exert their effect within the cell. Glycine is known to be taken up into frog CNS via an active transport mechanism (Davidoff & Adair, 1976) with two distinct saturable uptake systems, and more recently has been shown to induce catecholamine release from rat brain (Schmidt & Taylor, 1990). We (Yadid, Zinder & Youdim, unpublished observations) have found two glycine uptake sites on chromaffin cells the kinetic constants of which are $K_{m1} = 0.6 \times 10^{-6}$ M and $K_{m2} = 1.4 \times 10^{-3}$ M. These are almost identical to those found in the CNS for this amino acid (Logan & Snyder, 1972; Hannumiemi & Oja, 1981; Siemers *et al.*, 1982; Hardy *et al.*,

1986), indicating similarity between the central and peripheral cells in the functional characteristics of this amino acid.

However, these results do not exclude the possibility that the binding of glycine to specific receptors on the plasma membranes triggers an intracellular cascade of events culminating in catecholamine secretion. More recently we have shown in adrenal medulla chromaffin cells (Yadid *et al.*, 1989) the presence of a high affinity binding site for [³H]-strychnine ($K_d = 6.25$ nM), a glycine antagonist (Curtis *et al.*, 1971). This finding supports the evidence for the existence of glycine receptor activity (Yadid *et al.*, 1989) on these cells similar to that seen in the brain (Young & Snyder, 1974).

Since glycine, like GABA, is an inhibitory amino acid, it seems reasonable to suggest that glycine may parallel the activity of GABA, and induce secretion from the chromaffin cell (Kitayama *et al.*, 1986). It is also possible that the stimulation of release of catecholamines by glycine from bovine isolated chromaffin cells might be partially explained through its binding to a subunit of the acetylcholine receptor, perhaps the one shown by Grenningloh *et al.* (1987) to be homologous with the strychnine-sensitive subunit of the glycine receptor in the CNS. This could result in activation of the secretory cascade via the glycine receptor interaction with the nicotinic receptor.

In conclusion, the present findings demonstrate that uptake of glycine into the chromaffin cell is an effect seen to accompany the secretion of catecholamines. These results point to a possible peripheral role for glycine, perhaps in the modulation of catecholamine secretion from the adrenal medulla. The delivery of glycine into the adrenal medulla cell by a specific uptake mechanism or via the action of milacemide, or, alternatively, its binding to a specific membrane bound glycine receptor (Yadid *et al.*, 1989), can also serve as an excellent model for elucidation of the mechanism involved in the action of the inhibitory amino acid neurotransmitter, glycine, in the central nervous system. It remains to be seen whether milacemide releases catecholamines from brain structures in a similar manner. This latter possibility is supported by recent studies of Schmidt & Taylor (1990) and Mangano *et al.* (1990) who have shown the ability of glycine to release [³H]-adrenaline from superfused rat hippocampal brain slices.

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