Effect of proteolytic cleavage on functional properties of muscarinic acetylcholine receptors in rat pancreatic and parotid acinar cells

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Muscarinic acetylcholine receptors in isolated rat pancreatic acinar cells have an apparent M_r of 88 000, which could be decreased to 46 000 by papain, as deduced by covalent binding of the specific alkylating agent [^3H]propylbenzylcholine mustard. Muscarinic receptors on papain-treated acinar cells retained the antagonist-binding site and both high- and low-affinity binding sites for the cholinergic agonist carbachol. Similar results were observed in studies with rat parotid acinar cells, although the receptors in both control and papain-treated cells were each 10 000–15 000 Da smaller than in pancreas. Additionally, muscarinic receptors in papain-treated pancreatic acinar cells retained the ability to mediate carbachol stimulation of digestive-enzyme secretion. These results demonstrate that the characteristic binding properties of muscarinic receptors for both agonists and antagonists as well as their ability to translate agonist occupancy into a physiological response are not altered by proteolytic cleavage.

INTRODUCTION

We recently used the alkylating reagent [^3H]PrBCM, which specifically labels muscarinic receptors (Birdsall *et al.*, 1979; Venter, 1983; Peterson *et al.*, 1984), to probe their molecular structure in intact acini prepared by collagenase digestion of rat pancreas and lacrimal and parotid glands (Hootman *et al.*, 1985). We found that the apparent M_r of the receptor varied considerably. In rat pancreatic acini, a protein of apparent M_r 118 000 was labelled, whereas labelling of parotid and lacrimal acini gave estimates of receptor M_r of 105 000 and 87 000 respectively. The presence of the reducing agent β -mercaptoethanol during detergent solubilization of acini had no effect on the apparent M_r of the labelled proteins. When labelling was carried out in the presence of an excess of the competing antagonists atropine, QNB or scopolamine, alkylation by [^3H]PrBCM was blocked, demonstrating that the labelled proteins did in fact represent muscarinic receptors. Besides these primary labelled species, proteins of lower apparent M_r also were labelled. For all these tissues, a secondary peak of radioactivity usually was observed on gels at an M_r approx. 30 000–35 000 lower than the first. When dispersed acinar cells rather than acini were prepared from each of the three tissues by a procedure that involved digestion with a mixture of crude collagenase, hyaluronidase and α -chymotrypsin, and then labelled with [^3H]PrBCM, virtually all radioactivity was shifted into these secondary peaks and labelling of the primary peaks was abolished. Treatment of acini with α -chymotrypsin alone after [^3H]PrBCM labelling similarly shifted all label into these secondary peaks. The smaller receptor form thus appeared to be a fragment of the intact receptor. In a few experiments during these studies we also found that exposure of acinar cells to papain had an even more marked effect on the M_r of muscarinic

receptors. Digestion of acinar cells from lacrimal or parotid glands or pancreas with 1 mg of papain/ml after [^3H]PrBCM labelling decreased the apparent size of the receptors to M_r 40 000–50 000 in each case. Thus, for example, in intact pancreatic acinar cells, muscarinic receptor size could be decreased by up to two-thirds (i.e. from M_r 118 000 to 45 000) without losing the site to which [^3H]PrBCM covalently binds.

The ability selectively to diminish muscarinic receptors in intact acinar cells to various sizes by enzymic digestion has allowed us to ask questions regarding the relationship of receptor structure to function. Specifically, we have attempted to determine whether in acinar cells fragments of the muscarinic receptor display both high- and low-affinity agonist-binding states and retain the ability to mediate agonist stimulation of physiological responses. Our results indicate that muscarinic receptors in rat pancreatic and parotid acinar cells can be enzymically cleaved to an M_r as low as 40 000–50 000 and still display both the high- and low-affinity agonist-binding sites and retain the ability to translate receptor occupancy into a secretory response.

EXPERIMENTAL

Materials

Purified collagenase (448 units/mg) was purchased from Cooper Biomedical (Malver, PA, U.S.A.). Crude collagenase (160 units/mg), α -chymotrypsin (62 units/mg), deoxyribonuclease type 1 (800 units/mg), hyaluronidase (300 units/mg), papain (24 units/mg), trypsin inhibitor and carbamoylcholine chloride (carbachol) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine serum albumin (fraction V) was obtained from Miles Laboratories (Elkhart, IN, U.S.A.). Minimal Eagle's medium amino acids (50 $\times$ concentrate)

Abbreviations used: PrBCM, propylbenzylcholine mustard; QNB, quinuclidinyl benzilate; HR, Hepes-buffered Ringer.

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was from Grand Island Biological (Grand Island, NY, U.S.A.). QNB was obtained from Hoffman-La Roche (Nutley, NJ, U.S.A.). All other chemicals used were of reagent grade. L-[benzyl-4,4'- ^3H]Quinuclidinyl benzilate (30.2 Ci/mmol), [propyl-2,3- ^3H]propylbenzilylcholine mustard (43.3 Ci/mmol), Omnifluor and Protosol were purchased from New England Nuclear (Boston, MA, U.S.A.). Male Sprague-Dawley rats were purchased from Simonsen (Gilroy, CA, U.S.A.) and maintained on standard laboratory chow and water.

Preparation of acinar cells

Rats were killed by cervical dislocation and exsanguination. The pancreas or parotid glands were removed and trimmed of adipose and connective tissue. Dispersed acinar cells were prepared as described previously (Hootman *et al.*, 1983). After isolation, acinar cells were suspended in HR, which contained 118 mM-NaCl, 4.7 mM-KCl, 1.0 mM- Na_2HPO_4 , 1.1 mM- MgCl_2 , 0.5 mM- CaCl_2 , 5.5 mM-D-glucose, 10 mM-Hepes (sodium salt) and 2.0 mM-L-glutamine. This medium was supplemented with minimal Eagle's medium amino acids and adjusted to pH 7.4 with NaOH.

[^3H]PrBCM labelling and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of muscarinic receptors

Acinar cells were incubated for 30 min at 30 °C in HR containing 10 nM cyclized [^3H]PrBCM with or without 1 μM -QNB. After labelling, acinar cells were centrifuged at 50 g for 5 min, rinsed with 3 $\times$ 10 ml of HR and resuspended in 0.7 ml of HR, to which was added 0.3 ml of sample buffer at 95 °C, consisting of 9% (w/v) sodium dodecyl sulphate, 15% (v/v) glycerol, 0.05% (w/v) Bromophenol Blue and 30 mM-Tris/HCl (pH 7.4). Samples were heated at 100 °C for 5 min and, after cooling, applied to 3 mm-thick 7.5% (w/v) polyacrylamide gels. Protein in samples was determined by a Coomassie Blue binding assay (Bradford, 1976) with bovine serum albumin as standard. M_r standards (Bio-Rad SDS PAGE HIGH) were run simultaneously. After electrophoresis, strips containing M_r standards were sliced from gels and stained with 0.5% (w/v) Coomassie Blue in 50% (w/v) trichloroacetic acid. The remainder of the gel was cut into 3 mm slices (approx. 40/gel) and extracted for 24 h in a cocktail consisting of 1.0 ml of Protosol and 10 ml of Omnifluor before counting for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer.

To assess the effects of papain digestion on muscarinic receptors, acinar cells, after labelling with [^3H]PrBCM, were rinsed as described above to remove unbound ligand and resuspended in 10 ml of HR with or without 1.0 mg of papain/ml. Cells were incubated for 60 min at 37 °C and then pelleted, resuspended in 0.7 ml of HR, and solubilized and electrophoresed.

Effects of papain digestion on binding of [^3H]QNB and carbachol

Acinar-cell suspensions were prepared and incubated for 60 min at 37 °C in 10 ml of HR with or without 1.0 mg of papain/ml. Cells were then pelleted and resuspended in HR to a concentration of 2×10^6 /ml. For saturation binding studies, 5.0 ml volumes of this suspension were incubated for 120 min at 37 °C with concentrations of [^3H]QNB from 10 to 300 pM. Duplicate suspensions that also contained 10 μM -atropine were incubated in parallel

Fig. 1. Effect of papain digestion on apparent M_r of [^3H]PrBCM-labelled protein from dispersed rat pancreatic acinar cells

Cells were labelled and digested as described in the Experimental section. Each profile is representative of seven experiments. Protein standards run with each gel are: (1) myosin, M_r 200000; (2) β -galactosidase, M_r 116250; (3) phosphorylase b, M_r 92500; (4) bovine serum albumin, M_r 66200; (5) carbonic anhydrase, M_r 31000. The sample of protein from control cell suspension ($\bullet$) was 3.0 mg and that from papain-treated cells ($\circ$) was 2.5 mg.

at each QNB concentration for assessment of non-specific binding. At 120 min, acinar cells were collected on GF/A glass-fibre filters, rinsed with 3 $\times$ 5.0 ml of ice-cold 0.9 % NaCl, and extracted and counted for radioactivity as above. Carbachol-inhibition experiments were carried out by incubating 5.0 ml volumes of acinar-cell suspension with 100 pM-[^3H]QNB and concentrations of carbachol from 0.1 μM to 5.0 mM for 120 min at 37 °C before filtering, rinsing and counting for radioactivity as above. Scatchard analyses of saturation binding curves and fitting of curves for carbachol inhibition of [^3H]QNB binding were carried out with computer programs as described previously by Sankaran *et al.* (1982).

Effect of papain digestion on amylase secretion

Acinar cells were prepared and incubated as above in HR with or without papain, pelleted, and resuspended in HR. Amylase release in response to different concentrations of carbachol was determined as described previously by Williams *et al.* (1978), but with Procion Yellow-starch (Jung, 1980) as substrate. Amylase release over a 30 min incubation period was expressed as a percentage of total cellular amylase content at the beginning of the assay.

Expression of results

Experiments were repeated three to seven times, and results are expressed as means $\pm$ S.E.M.

Fig. 2. Scatchard plots of specific saturation binding curves of $[^3\text{H}]\text{QNB}$ to control (●) and papain-treated (○) acinar cells

Points represent the means of four experiments. Acinar cells were incubated in HR for 120 min at 37 °C with concentrations of $[^3\text{H}]\text{QNB}$ from 10 to 300 pM.

RESULTS

Effect of papain digestion on $[^3\text{H}]\text{PrBCM}$ labelling of dispersed pancreatic acinar cells

Electrophoresis of dispersed pancreatic acinar cells after labelling with $[^3\text{H}]\text{PrBCM}$ revealed a single peak of radioactivity corresponding to an M_r of $88\,300 \pm 3\,800$ (Fig. 1). This labelled peak was abolished when cells were preincubated with 1 μM -QNB or -scopolamine (results not shown). The position of this peak on gels was virtually identical with that of the secondary peak seen on gels of $[^3\text{H}]\text{PrBCM}$ -labelled pancreatic acini (Hootman *et al.*, 1985), although there was no evidence of the peak at M_r 120 000. When singly dispersed acinar cells were incubated for 60 min at 37 °C in HR containing 1 mg of papain/ml (24 units/mg) after labelling with $[^3\text{H}]\text{PrBCM}$, the labelled peak on gels was shifted to a position corresponding to an M_r of $46\,100 \pm 3\,100$ (Fig. 1). Lower concentrations of papain also were tested, but gave an incomplete shift of M_r of the labelled peak. This peak on gels of papain-digested acinar cells was characteristically quite broad and somewhat flattened compared with that seen with undigested controls, indicating some variability in the site(s) of proteolytic attack on the receptor by papain. Although not shown here, when pancreatic acini rather than single acinar cells were digested with papain after $[^3\text{H}]\text{PrBCM}$ labelling, some muscarinic receptors were decreased to M_r 40 000–50 000, although a substantial amount of radioactivity remained in the two higher- M_r peaks. This may reflect the inaccessibility of receptors to papain owing to the close apposition of basolateral plasma membranes in adjacent acinar cells in the intact acinus. By contrast, the entire cell surface is

Fig. 3. Carbachol displacement of $[^3\text{H}]\text{QNB}$ specifically bound to control (●) and papain-treated (○) pancreatic acinar cells

Cells were incubated in HR with 100 pM- $[^3\text{H}]\text{QNB}$ with or without carbachol for 120 min at 37 °C. Results represent the means of five experiments. Curves were generated by computer fitting to a two-site model.

freely accessible in singly dispersed pancreatic acinar cells.

Effect of papain digestion on $[^3\text{H}]\text{QNB}$ and carbachol binding to muscarinic receptors

From polyacrylamide gels of $[^3\text{H}]\text{PrBCM}$ -labelled acinar cells, papain appeared to decrease the apparent M_r of muscarinic receptors without decreasing the total amount of bound ligand. To quantify receptor numbers accurately, we incubated dispersed pancreatic acinar cells with papain as before and carried out saturation binding studies with $[^3\text{H}]\text{QNB}$, which previously has been used to characterize muscarinic receptors in pancreas and other exocrine glands (Ludford & Talamo, 1980; Putney & Van De Walle, 1980; Larose *et al.*, 1981). Scatchard analyses of these data (Fig. 2) indicated that papain treatment had no appreciable effect on either the binding maxima (B_{max}) or equilibrium dissociation constant (K_d) for the QNB-receptor interaction. Papain-treated acinar cells bound 28.3 ± 3.5 fmol of QNB/ 10^6 cells, with a K_d of 46.7 ± 1.2 pM, whereas the B_{max} and K_d for untreated cells were 28.6 ± 2.0 fmol/ 10^6 cells and 50.4 ± 1.4 pM respectively.

We also assessed effects of papain digestion on the affinity of pancreatic-acinar-cell muscarinic receptors for the cholinergic agonist carbachol. Treated and control cells were incubated in HR with 100 pM- $[^3\text{H}]\text{QNB}$ and concentrations of carbachol of 0.1 μM –5.0 mM, with the results shown in Fig. 3. Papain treatment did not cause any noticeable difference in position of the carbachol competition curve. When the curves in Fig. 3 were fitted by an iterative computer program (Sankaran *et al.*, 1982), they each corresponded most closely to a two-site model. For control cells, 74% of the total receptor population was represented by high-affinity sites, with a K_H of 32 μM . The low-affinity sites, 26% of the population, bound carbachol with a K_L of 1.8 mM. In suspensions

Fig. 4. Effect of papain digestion on carbachol stimulation of amylase secretion by pancreatic acinar cells

Results represent the means $\pm$ S.E.M. for five experiments and are expressed as percentage of total cellular amylase released during a 30 min incubation at 37 °C. ●, Control; ○, papain-treated.

of papain-treated acinar cells, 77% of carbachol-binding sites were of high affinity ($K_H = 32 \mu\text{M}$) and 23% were of low affinity ($K_L = 1.7 \text{ mM}$). These results indicate that muscarinic receptors, at least in pancreatic acinar cells, can be decreased substantially in size without losing characteristic binding properties for both agonists and antagonists.

Effect of papain digestion on acinar-cell amylase release

To determine whether the observed decrease in apparent M_r perturbed the ability of muscarinic receptors to transduce agonist occupancy into a physiological response, we measured carbachol stimulation of amylase release in papain-treated and control acinar cells (Fig. 4). The carbachol dose-response curve in both treated and control cells was biphasic. From an unstimulated basal release of $2.98 \pm 0.78\%$, amylase secretion in control cells rose to $9.05 \pm 0.80\%$ at $10 \mu\text{M}$ -carbachol before declining at higher concentrations. Basal amylase release was higher in papain-treated acinar cells ($4.25 \pm 0.70\%$), whereas maximal release, which was attained at $3 \mu\text{M}$ -carbachol, was slightly lower ($8.35 \pm 0.95\%$).

Effect of papain digestion on [^3H]PrBCM and carbachol binding to parotid acinar cells

To extend the results of these studies of the effects of papain on pancreatic muscarinic receptors, we carried out a more limited series of experiments with isolated parotid acinar cells. When intact acini are prepared from rat parotid glands and labelled with [^3H]PrBCM, the major peak of radioactivity on gels appears at M_r

Fig. 5. Effect of papain digestion on apparent M_r of [^3H]PrBCM-labelled protein from dispersed rat parotid acinar cells

Protein standards were as in Fig. 1. Each profile is representative of four experiments. The sample of protein from both control (●) and papain-treated (○) acinar cells was 0.4 mg.

102000–105000. A subsidiary peak is also invariably observed at M_r 75000–80000 (Hootman *et al.*, 1985). In the present studies, when dispersed acinar cells were prepared instead, the peak of greater M_r was not observed, and binding was entirely to a smaller receptor fragment, of M_r 79800 ± 1300 (Fig. 5). As with suspensions of pancreatic acinar cells, papain treatment decreased by about 40000 the apparent M_r of muscarinic receptors (to 39400 ± 1300) and broadened the peak seen on gels. In [^3H]QNB-carbachol competition experiments, papain also failed to shift the competition curve appreciably (Fig. 6). Computer fitting of the two curves again gave two classes of agonist-binding sites. In control cells, high-affinity sites ($K_H = 27 \mu\text{M}$) represented 80% of the receptor population, whereas low-affinity sites ($K_L = 0.84 \text{ mM}$) were 20%. In papain-treated cells, high-affinity sites ($K_H = 27 \mu\text{M}$) accounted for 81% of binding, and low-affinity sites ($K_L = 0.95 \text{ mM}$) represented the remaining 19% of the receptor population. Although we did not carry out [^3H]QNB-saturation-binding studies on papain-treated and control parotid acinar cells, in the carbachol competition experiments equilibrium binding at 100 pM -[^3H]QNB without carbachol was $20.4 \pm 1.1 \text{ fmol}/10^6$ cells for control and $18.8 \pm 1.6 \text{ fmol}/10^6$ cells for papain-treated cells. Thus, as in pancreatic acinar cells, papain digestion did not markedly decrease [^3H]QNB binding.

DISCUSSION

How the molecular structure of muscarinic receptors relates to their characteristic interactions with agonists and antagonists is not well understood. Nor is it at all

Fig. 6. Effect of papain digestion on carbachol displacement of $[^3\text{H}]\text{QNB}$ binding to parotid acinar cells

●, Control; ○, papain-treated. Conditions of incubation were as in Fig. 3. Results represent the means of three experiments, and curves were drawn by computer fitting of data to a two-site model.

clear how binding of acetylcholine and its analogues to these receptors is translated into appropriate subcellular responses, such as increases in polyphosphoinositide hydrolysis and inhibition of adenylate cyclase activity. To address these questions, we have used a specific affinity label for muscarinic receptors, $[^3\text{H}]\text{PrBCM}$, to mark these proteins in intact acinar cells from mammalian exocrine glands. We previously showed that the apparent M_r of muscarinic receptors in three organs varied from 60000 to 120000, as deduced by covalent labelling of acinar cells with $[^3\text{H}]\text{PrBCM}$. Labelling of muscarinic receptors in intact rat pancreatic acini revealed a major peak on polyacrylamide gels at a M_r corresponding to 118000. This peak always was accompanied by a smaller labelled peak of M_r 88000. In the rat parotid gland, a similar pattern is observed, although the apparent M_r values of both the primary and secondary peaks were about 10000 lower. In both pancreas and parotid gland, when singly dispersed acinar cells rather than acini were prepared and labelled with $[^3\text{H}]\text{PrBCM}$, all radioactivity was shifted into the secondary peaks of lower M_r (Hootman *et al.*, 1985; Figs. 1 and 5). The agent responsible for this reduction is α -chymotrypsin, which is used in preparation of isolated acinar cells, but not of acini. Additionally, when acini were pre-labelled with $[^3\text{H}]\text{PrBCM}$ and then treated with α -chymotrypsin, the peak of higher M_r on polyacrylamide gels was decreased in size to that of the lower M_r peak. However, α -chymotrypsin was not able to decrease muscarinic receptors further below this size in either pancreas or parotid acinar-cell suspensions. By contrast, papain was able to cleave another fragment from the receptor, so that the $[^3\text{H}]\text{PrBCM}$ -labelled fragment was decreased to M_r 40000–45000. This fragment was retained by the cells. Treatment of $[^3\text{H}]\text{PrBCM}$ -labelled pancreas and parotid acinar cells with papain did not release radioactivity into the medium

(results not shown), nor did it decrease maximal binding of $[^3\text{H}]\text{QNB}$ to the cells (Fig. 2). These results differ from those of Venter (1983), who treated $[^3\text{H}]\text{PrBCM}$ -labelled rat brain membranes with papain and thereby released a labelled subfragment of M_r 42000. These results indicate that up to half of the brain muscarinic receptor is outside the lipid bilayer. The reason why papain cleaves the antagonist-binding site from brain membranes, but not from intact pancreatic and parotid cells, is unclear. Slight differences in structure between epithelial and central-nervous-system receptors could render different sites accessible to papain. Or the difference may lie in using intact cells rather than membrane fractions as material for labelling and proteolysis. Either could conceivably result in generation of somewhat different fragments of the receptor. In the present studies, we did not digest $[^3\text{H}]\text{PrBCM}$ -labelled pancreatic and parotid plasma-membrane fractions with papain, since we have been interested primarily in the effect of proteolytic diminution of muscarinic receptors on subsequent physiological responsiveness, which necessitates the use of viable cells.

In intact pancreas and parotid-gland acinar-cell suspensions, the fragment cleaved by papain from that bearing the antagonist-binding site of the muscarinic receptor cannot be recognized by the present techniques, since it is unlabelled. Thus we do not know whether it is released into the medium or retained in the plasma membrane. If much of the muscarinic receptor resides in the extracellular environment, as the studies of Venter (1983) indicate, it most probably is released. If, however, the unlabelled muscarinic receptor fragment is retained in the plasma membrane, the two fragments may continue to associate within the bilayer, although on the basis of Venter's (1983) data we consider this unlikely.

Since papain treatment fragmented the receptor without liberating $[^3\text{H}]\text{PrBCM}$, we asked whether agonist binding could still elicit a physiological response. The answer appears to be affirmative. In both pancreas and parotid acinar cells that had been digested with papain, carbachol proved to be an effective inhibitor of $[^3\text{H}]\text{QNB}$ binding (Figs. 3 and 6). Furthermore, computer analysis of these competition curves indicated that both low- and high-affinity agonist-binding sites were present on the papain-fragmented receptor. These data indicate that the transition from low- to high-affinity agonist-binding states does not depend on dimerization of receptor monomers, as was suggested by Avissar *et al.* (1982, 1983). Instead, it appears that the two classes of sites co-exist in a small fragment of the receptor that also contains the site to which antagonists bind. In addition, decreasing the size of muscarinic receptors on pancreatic acinar cells by papain did not prevent carbachol stimulation of amylase secretion (Fig. 4). However, basal amylase release was increased and the maximal response was attenuated, suggesting that papain treatment at this high dose caused generalized damage to the cell surface. Indeed, microscopic examination of treated acinar-cell suspensions revealed surface blebbing in many cells, although nuclear staining with Trypan Blue was not noticeably increased relative to control cells. In both cases, less than 10% of cells were permeable to the vital dye after incubation. Despite this evidence of the general deleterious effects of papain, the characteristic biphasic dose-response to carbachol was still observed, and the magnitude of the response was decreased by only about one-third. It is possible that this response was due to the

continued presence of a small number of M_r -80 000–90 000 receptors, since overlap in the [3 H]PrBCM-labelled peaks in Fig. 1 indicates that as much as 10% of the muscarinic receptor population could have escaped decrease in size by papain. However, Dehaye *et al.* (1984) demonstrated in rat pancreatic acini that amylase secretion in response to carbachol was unimpaired when up to 50% of muscarinic receptors were occluded by PrBCM, but, when 90% of the receptor population was alkylated by the antagonist, the magnitude of maximal amylase secretion was decreased by about one-half, and the concentration of carbachol needed for half-maximal stimulation was increased substantially. On this basis, we believe that retention of a small percentage of undigested muscarinic receptors on acinar cells cannot account for the secretory response after papain digestion. Since release of amylase represents the terminal step in a cascade of intracellular events triggered by receptor occupancy, which includes stimulation of polyphosphoinositide hydrolysis and the release of Ca^{2+} sequestered within the cell, we conclude that coupling of muscarinic receptors with primary effector molecules is not seriously impaired by this proteolytic cleavage. The form of the muscarinic receptor represented by the M_r -40 000–50 000 [3 H]PrBCM-binding protein must be competent to bind agonists and transduce the signal encoded in their chemical structure into a sequence of intracellular events that results in secretion of digestive enzymes. If this is so, however, it leaves open the question of the functional significance of the remainder of the receptor. The use of immunological reagents to probe the structure and biosynthesis of muscarinic receptors would seem to be a promising approach for further investigations.

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