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Relationships between chemical structure and inhibition of epinephrine-induced human blood platelet aggregation

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The effect of structural features in a series of carbamoylpiperidine and nipecotoylpiperazine congeners on epinephrine-induced aggregation of human blood platelets is examined. Epinephrine-induced *primary* aggregation is effectively inhibited by the nipecotoylpiperazine derivatives (culminating at an I_{PA50} of 11.9 μM). While among nipecotoylpiperazine as well as carbamoylpiperidine congeners there are potent inhibitors of ADP-stimulated platelet function (cresting at an I_{A50} of 12.4 and 11.4 μM, respectively), the carbamoylpiperidine analogs are much less active (e.g., I_{PA50} of 298.1), or practically inactive, in impeding epinephrine-induced *primary* aggregation (PA).

Introduction

We have been engaged in relating structural features of synthetic entities to their effect on human blood platelets, employing the compounds as molecular probes in endeavoring to further elucidate mechanisms of platelet function [1–19]. The tertiary amines (structured around the ring nitrogens)in our compounds, identified as aggregation-inhibitory specific functions [2], are subject to broad variances in protonation contingent upon the pH of their immediate vicinity and upon the specific compounds' pK_a values. This very trait enables them, in their specifically structured molecules [1–3,6,7,16], to assume appropriate hydrophobic character for the penetration of the platelet membrane's lipid bilayer without interfering, subsequently, with their transformation into corresponding cations [1,3,7,12]. Within this context, the penetrated amines in our surface active molecules can be envisioned to generate their cationic species in quantities capable of interacting with and reducing the response sensitivity of anionic phospholipids [5] (cf. Ref. 20). Our conclusions are supported by the findings of Sheetz and Singer [21] (cf. Ref. 22) and Ferrel et al. [23], even though these groups of investigators differ among themselves in regard to the morphological mechanisms effected by cationic amphipaths. In this manner, then, our compounds are capable of stabilizing membrane complexes of the dense tubular system (DTS) and of other storage sites sequestering calcium in the platelets [4,24]. By enhancing the integrity of these membrane complexes, the compounds should block or restrain Ca^{2+} release into the platelet's cytosol [4,15] upon stimulation sparked by energized receptors and, consequently, impede activation of phospholipase A_2 as well as the pathway associated with it [25]. Moreover, by reducing the response sensitivity of anionic phospholipids [5] (cf. Ref. 20), the compounds render phosphatidylinositol 4,5-bisphosphate (PIP_2) unsusceptible or less susceptible to hydrolysis by phospholipase C which, in turn, prevents or curtails generation of inositol 1,4,5-trisphosphate (IP_3) [26], perceived as the principal moiety to trigger internal discharge of Ca^{2+} [27] (cf. Ref. 28). Consequently, the threshold for triggering and/or sustaining platelet aggregation should be elevated, and only stimuli of considerably greater intensity could actuate the process [1–3,7–8,12,15].

In previous investigations we evaluated the influence of our compounds on human blood platelet aggregation effected by a number of inducing agents (see, for example, Refs. 3, 4, 8, 15). At this time, we examined in depth their action on epinephrine-stimulated platelets, and were particularly interested in identifying variances

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between their impact on the latter and on ADP-induced aggregation.

Materials and Methods

The test compounds used in this investigation have been designed and synthesized in our laboratories and were discussed in earlier publications [1–19]. They include: α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene dihydrobromide (I); 1,6-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]hexane dihydrobromide (II); 1,10-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]decane dihydrobromide (III); 1-decyl-3-(*N*,*N*-diethylcarbamoyl) piperidine hydrobromide (IV); *N*,*N*′-bis(1-decylnipecotoyl)piperazine dihydriodide (V); *N*,*N*′-bis(1-hexylnipecotoyl)piperazine dihydriodide (VI); 1,2-bis[*N*-(1-decylnipecotoyl)-*N*-methylamino]ethane dihydriodide (VII); and 1,2-bis[*N*-(1-hexylnipecotoyl)-*N*-methylamino]ethane dihydriodide (VIII).

In preparing a new batch of VI, we noted that the melting point for the old batch of the compound should have been recorded as 254.6–255.2°C and that of its quaternary intermediate, *N*,*N*′-bis(1-hexylnicotinoyl) piperazine diiodide, as 245.0–246.0°C. The new batch substantiated these values, and they supersede those reported earlier (Ref. 3, p. 85).

Similar observations were made in synthesizing supplemental amounts of compound V; the melting point for the old batch should have been recorded as 268.2–268.9°C. Again, the new batch corroborated this value, and it supersedes that reported earlier (Ref. 12, p. 50 (Table I)). The melting point of the quaternary intermediate, *N*,*N*′-bis(1-decylnicotinoyl)piperazine diiodide, remains unchanged. Also, in preparing additional quantities of VII and VIII, this time, we succeeded in obtaining the compounds in crystalline form with (i) toluene/ethyl acetate and (ii) toluene/methyl ethyl ketone solvent systems, respectively. In their crys-

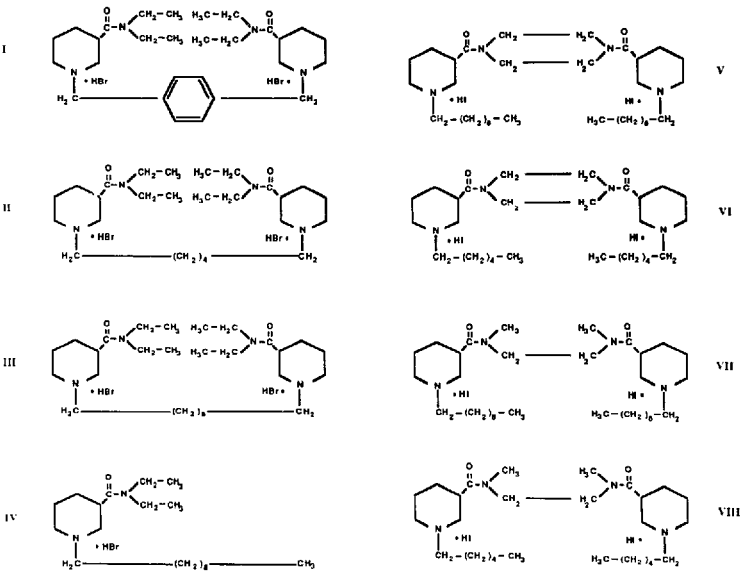

Fig. 1. Relationships in the structural features of carbamoylpiperidine and nipecotoylpiperazine congeners employed in the study.

talline form, they melted at 207.0–207.9 °C and at 198.1–199.0 °C, respectively. The identity of both compounds was, again, analytically confirmed (Galbraith Laboratories, Knoxville, TN). These values supersede those reported earlier (Ref. 12, p. 50 (Table I)).

Our reference compounds, yohimbine hydrochloride (R-I) (Cat. No. Y-3125, Lot No. 86F-0717) and chlorpromazine hydrochloride (R-II) (Cat. No. C-8138, Lot No. 71F-7704), were both procured from Sigma Chemical Co., St. Louis, MO.

Prior to aggregometric evaluation, compounds were normally dried at 100 °C for 0.5 h at 0.030–0.010 mmHg, and stored in a desiccator over Drierite (protected from light) until use. R-I, however, was dried 5 h at 0.075–0.009 mmHg at room temperature (26–27 °C).

The use of adenosine 5'-(trihydrogen diphosphate) (ADP), in the form of its sodium salt (anhydrous, 95–99% pure, essentially vanadium-free grade, Cat. No. A-6521, Lot No. 46F-7285, Sigma Chemical Co., St. Louis, MO), as a human blood platelet aggregation-inducing agent was described earlier [3,8,14]. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by centrifugation, simultaneously, in a Beckman J6-B ($120 \times g$, for 15 min) and in a Sorvall GLC-2B ($1400 \times g$, for 18 min), both at room temperature, respectively. This enabled us to initiate control/treated PRP aggregation runs, including 4 min preincubation and a 5.5 min tracing phase, at 60/61, 70/71 and 80/81 min postvenipuncture; the figures obtained in each of these three runs were averaged into single values. Plastic pipette tips with Centaur pipettes and polystyrene, polycarbonate or polypropylene tubes were employed throughout, except for siliconized glass cuvettes with siliconized stir bars used in the Payton dual channel aggregation module. The plasma was kept at 37 °C and the pH maintained within the appropriate range by displacing gently (2 min) the air above it with a 5% CO_2/95% air v/v mixture. Retention of CO_2 (5% CO_2/95% air) in the vials after initial gas displacement was achieved by keeping them, as well as aggregometer cuvettes, covered with Parafilm throughout the protocol whenever the procedure enabled us to do so. The pH of samples from untreated and unstimulated PRP in each set of aggregometric runs was monitored at 62 and 82 min after venipuncture; readings ranged from 7.51 ± 0.004(S.E., $n = 102$) to 7.52 ± 0.004(S.E., $n = 102$). In employing the other aggregation-inducing agent, (−)-epinephrine in the form of its (+)-bitartrate salt (Cat. No. E-4375, Lot No. 37F-0423, Sigma Chemical Co., St. Louis, MO) was added in 50 μl of aqueous 0.9% NaCl to 0.45 ml of PRP to yield a final concentration of 10 μM epinephrine.

All other reagents, methodology and the turbidimetric procedure employed in the aggregometric determinations were also reported previously [3,8,14]. Again, blood was acquired from different donors for each of the, at least, duplicate independent runs. They had fasted overnight and had abstained from all medications, alcohol, tobacco and caffeine for at least one week prior to donations. Through all experiments the age of the 12 healthy male volunteers ranged from 20 to 36 years, and the minimal concentration of ADP eliciting maximal biphasic aggregation averaged 5.4 ± 0.4(S.E.) μM for 22 plasma samples. We continually monitored the time frame from the inception to the termination of the primary wave generated by epinephrine, in tracings of the control runs (82.5 ± 1.5(S.E.) s, 80 determinations), and projected the termination point from the control tracing onto the one generated by the test-compound-containing PRP; this enhanced substantially the reproducibility and the accuracy of our values.

As indicated earlier [14] the values for the 50% inhibition of platelet aggregation (I_{A50}) were derived from the least squares analysis of points representing the means of inhibitory activities of at least two independent determinations for each concentration. Individual determinations of % inhibitory activity, at a given concentration, normally did not vary from computed values by more than ± 5 percentage points and frequently less.

Results

The data generated in this investigation are summarized in Table I, structured to accentuate the analogy and divergence between the compounds' effect on epinephrine-stimulated platelets and ADP-induced aggregation. To broaden characterization of the impact our compounds exert on epinephrine-stimulated platelets, we identified concurrently the compounds' effect on *total* aggregation (I_{TA_T}) at the concentration determined to be its I_{PA50} value for inhibiting *primary* aggregation (Fig. 2, Table I). The terms primary and

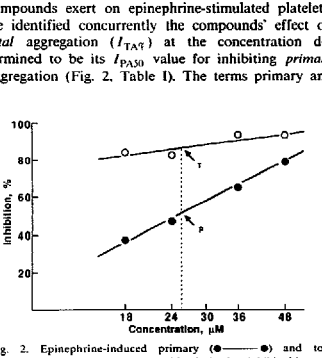

Fig. 2. Epinephrine-induced primary (●——●) and total (○——○) aggregation of human blood platelets inhibited by compound VII. Inhibition of *total* (T) aggregation (I_{TA_T}) corresponding to the I_{PA50} for *primary* (P) aggregation. I_{PA50} 25.8 μM (slope, 93.1); I_{TA_T}, 86.1 (slope, 24.3).

TABLE I

Aggregation-inhibitory potency of carbamoylpiperidine and nipecotoylpiperazine congeners

Compound	Epinephrine-induced		ADP-induced
	Primary aggregation I_{PA50} (μM)	Corresponding total aggregation I_{TA9}	Total aggregation I_{A50} (μM) [a]
I	[b]	[b]	11.4
II	[b]	[b]	64.3
III	[b]	[b]	23.5
IV	298.1	85.9	174.3
V	11.9	89.9	12.4
VI	78.5	82.9	54.7
VII	25.8	86.1	24.0
VIII	63.3	88.5	18.0
R-I	0.23	[c]	[d]
R-II	71.3	84.8	73.1

[a] From Lassło et al. [14].

[b] Compounds I, II and III did not effect 50% inhibition of epinephrine-induced *primary* aggregation at concentrations up to 22.8–91.2 μM, 64.3–514.4 μM and 120.0–240.0 μM, respectively (at these concentrations, they registered 77.1 ± 2.9(S.E., $n = 6$) to 85.2 ± 1.1(S.E., $n = 6$) %, 72.7 ± 0.4(S.E., $n = 6$) to 82.1 ± 2.0(S.E., $n = 6$) % and 77.6 ± 2.4(S.E., $n = 6$) to 85.9 ± 0.8(S.E., $n = 6$) % inhibition of epinephrine-induced *total* aggregation, correspondingly).

[c] At concentrations below 0.20 μM, yohimbine did not elicit secondary aggregation and, therefore, a percentage for the compound's inhibitory effect in *total* aggregation corresponding to its I_{PA50} value is not available.

[d] At concentrations 0.23–23.0 μM no inhibition of ADP-induced *total* aggregation was discerned.

total aggregation have been employed in accordance with the definitions articulated by Ding et al. [29] and also applied by Emanuelli et al. [30].

The value listed for compound VIII under ADP-effected aggregation (I_{A50}, 18.0 μM) does not reflect the trend of enhanced inhibition with increasing hydrophobicity registered by compounds VI and V (I_{A50}, 78.5 μM and 11.9 μM), and VIII and VII (I_{A50}, 63.3 μM and 25.8 μM), in their effect on epinephrine-induced *primary* aggregation, and by VI and V (I_{A50}, 54.7 μM and 12.4 μM) on ADP-stimulated platelets. To make certain that this value we reported for compound VIII earlier [14] is correct, we redetermined its I_{A50} in ADP-induced aggregation and confirmed its validity (I_{A50}, 21.2 μM). In the process we discerned, in a total population of 12 donors, five high-responders (I_{A50}, 14.6 μM) and five low-responders (I_{A50}, 27.7 μM) with the greatest divergence at lower, and increasing convergence at higher, concentrations.

In regard to the reference compounds, as indicated in Table I, yohimbine did not register any inhibition of ADP-stimulated platelets at concentrations up to 100-fold higher than those effecting 50% inhibition of epinephrine-induced *primary* aggregation.

Discussion

Considerable differences exist in the respective mechanisms of action associated with ADP- and epinephrine-triggered aggregations. There is, however, a distinct common denominator. While ADP is the dominant agonist in exposing fibrinogen binding sites, epinephrine can effect formation of interplatelet fibrinogen bridges (in the absence of discernible quantities of ADP) but to a much lesser extent [31] (cf. Refs. 32, 33). Epinephrine's ability to effect human blood platelet aggregation has been overwhelmingly linked to its stimulation of α_2-adrenoceptors [34–36] and Tremblay and Hamet [37] contend that in human platelets, due to the density of α_2-adrenoceptors, it inhibits adenylate cyclase subsequent to its occupancy of the receptor. The consequent reduction in basal and/or previously stimulated levels of cAMP results in a lack of natural means (a) for reducing cytosolic Ca^{2+} by redistributing the free calcium into platelet vesicle storage sites, and (b) for rendering less accessible pivotal platelet membrane sites for the platelet aggregation process [1]. Powling and Hardisty [38] found recently that epinephrine enhances intracellular mobilization, as well as influx, of extracellular Ca^{2+}. Most recently, emphasis has been placed on receptor-accelerated Na^+/H^+ exchange which, by alkalinizing the platelet interior, sensitizes phospholipase A_2 to ambient Ca^{2+} concentrations [39] (cf. Ref. 40). In any event, the premise associating the primary wave with the agonist's binding to α_2-receptors, in epinephrine-induced aggregation, remains undisputed [41].

As we observed in our previous studies [1–3,5–8], molecular features imparting hydrophobic characteristics can have substantial influence on the compounds' platelet aggregation-inhibitory activity. Here, too, the molecules' hydrophobic profile was clearly reflected in the impact exerted by nipecotoylpiperazine congeners on the *primary* phase of epinephrine-induced aggregation. Compounds VII and VIII match compounds V and VI, respectively, in all aspects except for one missing bond between two carbons of the piperazine moiety linking the latter, which differentiates the congeners into 1,2-bis(methylamino)ethane analogs and piperazine derivatives, correspondingly (Fig. 1). The 1-decyl substituted nipecotoylpiperazine congener (V) was more than 6-fold stronger than its 1-hexyl substituted analog (VI); similarly, 1,2-bis[*N*-(1-decylnipecotoyl)-*N*-methylamino]ethane (VII) was decidedly more potent than its corresponding 1-hexylnipecotoyl derivative (VIII) (Table I). Variations in potencies notwithstanding, one is tempted to conjecture that all these analogs could be capable of substantially interfering with epinephrine's binding to α_2-adrenoceptors in view of the work by Mehta et al. [41], demonstrating decisive correlation between binding of (−)-epinephrine to platelet α_2-

adrenoceptors and the agonist's ability to elicit *primary* wave aggregation response.

Since compounds VI and VIII differ from V and VII *solely* by having four fewer methylene units in their respective alkyl substituents on the ring nitrogens (Fig. 1), it is reasonable to conclude that increase in hydrophobicity enhances inhibitory potency in epinephrine-induced *primary* aggregation. This parallels the trend observed for VI and V in ADP-induced *total* aggregation [16] (Table I); the fact that VIII and VII exert inhibition at around the same level, in ADP-induced *total* aggregation, seems to be an exception and –since our compounds have been designed with gradual changes in their molecular constitution– can be attributed, in that specific set of congeners, to the severed bond in the linking moiety derived from piperazine (see Fig. 1, Table I and Results).

One could look upon compound V as the fusion of two molecules of compound IV which share the *same* two ethyl substituents on *each* of their respective amide nitrogens (Fig. 1). Compound IV has only a single aggregation-inhibitory specific function (tertiary amine structured around the ring nitrogen) compared to the two embodied within the molecule of compound V and is, in accordance with our previous postulates for ADP-induced aggregation [1,8], substantially less potent in inhibiting epinephrine-induced *primary* aggregation (25-fold) as well as ADP-induced *total* aggregation (14-fold) (Table I).

In a different vein, one could envision a fusion of two molecules of compound IV which share the *same* decyl substituent on *each* of their respective piperidino nitrogens. This configuration is attained in compound III (Fig. 1) and, since its molecule incorporates two aggregation-inhibitory specific functions, it should be more potent than compound IV with only a single aggregation-inhibitory specific function; and, indeed, compound III is over seven times more potent in inhibiting ADP-induced *total* aggregation [16]. We discerned a striking contrast, however, in the effect of compound III on epinephrine-induced *primary* aggregation. While registering an I_{A50} of 23.5 μM in ADP-induced *total* aggregation, it was unable to effect 50% inhibition of epinephrine-induced *primary* aggregation in concentrations up to 120–240 μM (at the referenced concentrations it exerted 77.6 $\pm$ 2.4(S.E., $n = 6$) to 85.9 $\pm$ 0.8(S.E., $n = 6$) % inhibition in epinephrine-induced *total* aggregation). Congeners of 1,10-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]decane (compound III), the corresponding hexane- and xylylene-linked bis(carbamoylpiperidino) derivatives (compounds II and I, respectively) registered analogous platelet aggregation-inhibitory profiles (Table I).

It is rather striking that, in a series of comparatively *closely* related congeners, a select few among quite potent inhibitors of ADP-induced aggregation (particularly I and III) lacked the modality to effect 50% inhibition of epinephrine-induced *primary* aggregation at manyfold higher concentrations (Table I). The nipecotoylpiperazine congeners (V, VI, VII and VIII) are quite effective in inhibiting epinephrine-induced *primary* aggregation as well as ADP-induced *total* aggregation. While the carbamoylpiperidine analogs with two aggregation-inhibitory specific functions (I, II and III) are potent in quelling ADP-induced *total* aggregation, they are not capable of impeding meaningfully epinephrine-induced *primary* aggregation.

Reducing the aggregation-inhibitory specific functions in the molecule from two (e.g., compound III) to a single one (compound IV), weakens substantially inhibition of ADP-induced *total* aggregation; concurrently, the compound (IV) acquires a limited but decidedly tangible inhibitory activity in epinephrine-induced *primary* aggregation. This occurs apparently because, in the absence of the second polar carbamoylpiperidine moiety, the singly substituted decane enhances the hydrophobic character of the molecule [6]. One may also conjecture that the flexibility of the remaining single aggregation-inhibitory specific function may render it more accessible for interference with (−)-epinephrine's binding to α_2-adrenoceptors since, as stated above, Mehta et al. [41] have shown decisive correlation between (−)-epinephrine's binding to α_2-adrenoceptors and its ability to effect *primary* aggregation. The same conjecture may be extended to the nipecotoylpiperazine congeners in which a corresponding flexibility (compounds V, VI, VII and VIII) and parallel increase in hydrophobicity (compounds V and VII) are apparent. They could be envisioned to be much more potent epinephrine-induced *primary* aggregation inhibitors, possibly because two aggregation-inhibitory specific functions, within a single molecule, are accessible for interference with the binding of (−)-epinephrine to α_2-adrenoceptors.

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