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Rapid intracellular release of calcium in human platelets by stimulation of 5-HT_2-receptors

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1 The concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in human blood platelets was measured by use of the fluorescent probe quin-2.

2 5-Hydroxytryptamine (5-HT) caused a rapid increase of $[Ca^{2+}]_i$ in the presence or absence of Ca^{2+} in the medium. The $[Ca^{2+}]_i$-rise was less marked in the absence of Ca^{2+} and could be antagonized by 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate-hydrochloride (TMB-8), an inhibitor of calcium release from internal stores.

3 5-HT induced a shape change reaction in the presence or absence of extracellular Ca^{2+}, but the pEC_{50} of 5-HT was slightly higher in the presence of the cation. Shape change reaction and $[Ca^{2+}]_i$-rise showed similar time courses.

4 Various 5-HT-agonists caused a rise of $[Ca^{2+}]_i$, whereas 5-HT-antagonists, but not the 5-HT-uptake inhibitor desmethylimipramine and the α_2-adrenoceptor antagonist yohimbine, counteracted the 5-HT-induced rise of the cation in a stereospecific manner. The antagonists were more potent than the agonists. The orders of potencies of the drugs affecting $[Ca^{2+}]_i$ and platelet shape were similar.

5 It is concluded that stimulation of 5-HT_2-receptors of platelets causes a rapid release of intracellular calcium which, by activation of the contractile system, mediates the shape change reaction.

Introduction

In previous work evidence has been presented that the shape change reaction (transition from the normally occurring discoid shape into a spheroid form with extrusion of pseudopods) induced in human platelets by 5-hydroxytryptamine (5-HT) is mediated by a rise in cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$). However, this increase was rather slow (maximum attained after 30 min) and was only seen in the presence of extracellular Ca^{2+} (Affoter *et al.*, 1984). In contrast, the 5-HT-induced shape change reaction is a rapid event which also occurs in a Ca^{2+}-poor medium containing disodium edetate (EDTA) (Born *et al.*, 1978; Graf & Pletscher, 1979; Affolter *et al.*, 1984).

Using a slightly modified quin-2-method we have shown in the present experiments that in platelets, stimulation of the 5-HT_2-receptor causes an elevation of intracellular free Ca^{2+} as rapid as the shape change reaction, even in a Ca^{2+}-poor medium.

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Methods

Measurement of $[Ca^{2+}]_i$

Citrated blood from healthy volunteers was obtained by vein puncture after overnight fasting and platelet-rich plasma (PRP) prepared by centrifugation as previously described (Erne *et al.*, 1984). The determination of $[Ca^{2+}]_i$ was carried out by use of the fluorescent probe quin-2, according to the method of Rink *et al.* (1982) slightly modified. The platelets were incubated in PRP at 37°C for 10 min with 15 μM (final concentration) quin-2 before separation by gel filtration, in contrast to previous experiments in which quin-2-loading of gel-filtered platelets was carried out for 30 min in a calcium-poor medium. Using this modification the intracellular content of quin-2 as determined by h.p.l.c. was less than 1 mM compared with 4 mM in previous experiments (Affolter *et al.*, 1984). Also the time needed for the Ca^{2+} to reach intracellular equilibrium after gel filtration of the platelets was markedly reduced (seconds instead of 15 min).

The incubation of the gel-filtered platelets was carried out in HEPES buffer (Erne *et al.*, 1984) with or without 1 mM Ca^{2+}, in the latter case in the presence of 5 mM EDTA. Drugs were added with a Hamilton syringe after registration of the basal fluorescence (F); the 5-HT-antagonists were added 10 s before 5-HT.

Measurements of the fluorescence were performed at 37 ± 0.5°C with slight stirring in an Aminco-Bowman spectrofluorimeter.

Calculations

The following formula was used:

$$[Ca^{2+}]_i = K_d\ (F - Fmin)/(Fmax - F).$$

K_d, the dissociation constant of quin-2-calcium, is 115 nM for the experimental conditions used (Tsien *et al.*, 1982). Fmin and Fmax stand for the maximal and minimal fluorescence at very low or high Ca^{2+}-concentration respectively after lysis of the platelets (for details see Erne *et al.*, 1984).

pEC_{50} and pIC_{50} are the negative logarithms of the ED_{50} and IC_{50} values. EC_{50} and IC_{50} are the molar concentration of agonists and antagonists, respectively, causing half maximal rise of $[Ca^{2+}]_i$ or 50% inhibition of the maximal $[Ca^{2+}]_i$ rise induced by 5-HT 10^{-6} M.

The concentration of calcium mobilized by 5-HT ($[Ca^{2+}]_{mobil}$) was calculated from the following formula:

$$[Ca^{2+}]_{mobil} = [Ca^{2+}{}_{HT}]_i - [Ca^{2+}{}_b]_i + [\text{quin-2}] \times \left(\frac{1}{1 + K_d/[Ca^{2+}{}_{HT}]_i} - \frac{1}{1 + K_d/[Ca^{2+}{}_b]_i} \right)$$

[Quin-2] is the concentration of the free acid in the platelets determined by h.p.l.c. (see Methods), $[Ca^{2+}{}_b]_i$ and $[Ca^{2+}{}_{HT}]_i$ represent the cytoplasmic free calcium under unstimulated (basal) and stimulated (5-HT) conditions respectively.

For statistical analysis Student's *t* test and the Mann-Whitney-U-test were used. Results are expressed as means with s.e.

Shape change

The shape change reaction of platelets was measured as previously described in the presence and absence of EDTA 5 mM, in both PRP and platelets isolated by a dextran gradient and resuspended in calcium-poor Tris buffer (Laubscher & Pletscher, 1979). Various concentrations of 5-HT were added after pre-incubation of the platelets for 10 min. An Elvi aggregometer was used for the measurements.

Materials

Quin-2-AM (2-methyl-6-methoxy-8-nitroquinoline tetraacetoxymethylester) and psilocin were kindly provided by SANDOZ Inc. Basel and D- and L-butaclamol by F. Hoffmann-La Roche Inc. Basel, Switzerland. The quin-2-AM showed a more than 96% purity as checked by reversed phase high performance liquid chromatography (h.p.l.c.). The impurity consisted mainly of the free acid, partially hydrolyzed quin-2-AM could not be detected. All other chemicals were of analytical grade and obtained from commercial sources.

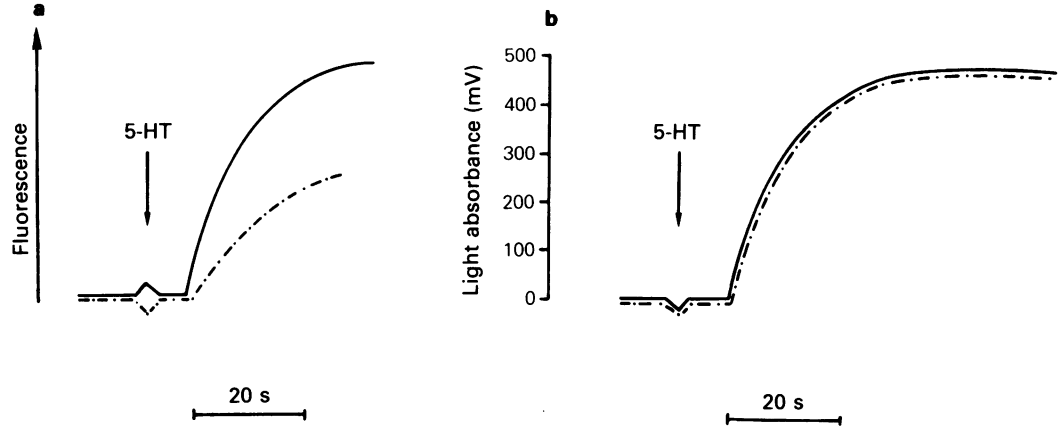

Figure 1 (a) Effect of 5-hydroxytryptamine (5-HT) on intracellular free calcium ($[Ca^{2+}]_i$) in platelets. Increase in fluorescence in quin-2-loaded platelets in the presence of 1 mM Ca^{2+} in the medium (continuous line). Platelets from the same donors, but with 5 mM EDTA instead of Ca^{2+} in the medium (broken line). Arrows indicate addition of 5-HT (10^{-6} M). (b) Shape change reaction induced by 5-HT 10^{-6} M in platelet-rich plasma in the presence (continuous line) and absence (broken line) of EDTA 5 mM. Each trace is a typical experiment.

Results

Cytoplasmic free Ca^{2+}

The basal concentration of $[Ca^{2+}]_i$ in the platelets was somewhat lower in the absence than in the presence of extracellular Ca^{2+}. 5-HT (10^{-6} M) caused a rapid and significant ($P < 0.01$) rise of $[Ca^{2+}]_i$ which was maximal after 25 s and occurred both in the presence and absence of Ca^{2+} in the medium (Figures 1 and 2). In the presence of extracellular Ca^{2+} the increase in $[Ca^{2+}]_i$ was more pronounced than in its absence (Figures 1 and 2). In the absence of extracellular Ca^{2+} the intracellular concentration of calcium mobilized by 5-HT ($[Ca^{2+}]_{mobil}$) was as high as 9.5×10^{-5} M. 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) and ketanserin abolished the 5-HT-induced rise of cytoplasmic $[Ca^{2+}]_i$ in platelets incubated in Ca^{2+}-free medium (Figure 3).

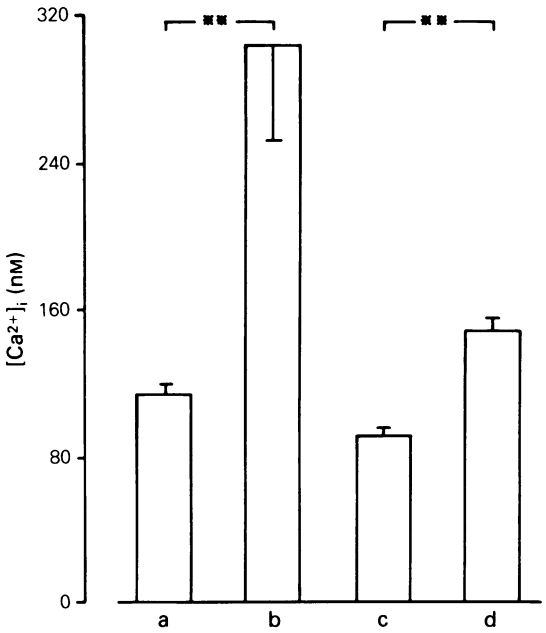

Figure 2 Effect of 5-hydroxytryptamine (5-HT) on intracellular free calcium ($[Ca^{2+}]_i$). (a) Non-stimulated platelets in medium containing 1 mM Ca^{2+}. (b) Platelets treated with 10^{-6} M 5-HT in the same medium as in (a). (c) Unstimulated platelets in Ca^{2+}-poor medium containing 5 mM EDTA. (d) Platelets treated with 10^{-6} M 5-HT in the same medium as in (c). Measurements were performed 25 s after addition of 5-HT. The columns are means with s.e. of 7 independent experiments. $^{**}P < 0.01$.

Effects of drugs

The experiments were mainly carried out in the presence of extracellular Ca^{2+}, since the 5-HT-induced rise in $[Ca^{2+}]_i$ was more marked with Ca^{2+} in the medium than without and since 5-HT and ketanserin exhibited their action in both conditions (Figure 3, Table 1).

Various 5-HT-agonists caused a rise of cytoplasmic free Ca^{2+}, whereas the 5-HT-antagonists counteracted the increase of $[Ca^{2+}]_i$ due to 10^{-6} M 5-HT (Table 1). The effects of 5-HT, 5-HT-agonist and -antagonist were dose-dependent (Figure 4), the 5-HT-antagonists showing more marked potency than the agonists. The inhibitory action of butaclamol was stereospecific, the D-form being two orders of magnitude more potent than the L-enantiomer. Desmethylimipramine (DMI), an inhibitor of the 5-HT-uptake at the plasma membrane, and yohimbine, an α_2-adrenoceptor antagonist, did not prevent the 5-HT-induced $[Ca^{2+}]_i$-rise (Table 1).

Shape change

In PRP 5-HT (10^{-6} M) caused a rapid shape change reaction, which was maximal after about 30 s and occurred in the presence and absence of EDTA 5 mM (Figure 1). However, the pEC_{50} of 5-HT was slightly, but significantly higher in the absence (7.09 ± 0.04) than in the presence (6.84 ± 0.03, $P < 0.05$) of EDTA ($n = 6$, PRP). In platelets isolated by a dextran gradient and suspended in an artificial medium devoid of Ca^{2+} and containing 5 mM EDTA, 5-HT 10^{-6} M also caused a typical shape change reaction.

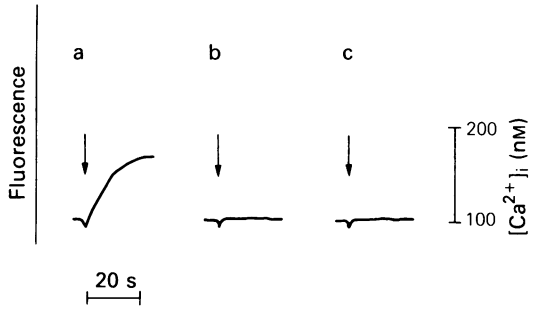

Figure 3 Effect of ketanserin and TMB-8 on the 5-hydroxytryptamine (5-HT)-induced rise of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in the absence of Ca^{2+}, but in the presence of 5 mM EDTA in the medium. Arrows indicate addition of 5-HT 10^{-6} M. (a) 5-HT alone; (b) 5-HT 10 s after ketanserin 10^{-6} M; (c) 5-HT 10 s after TMB-8 10^{-6} M. Results from a single experiment.

Table 1 Effect of various drugs on the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) and on platelet shape

Drug	n	$[Ca^{2+}]_i$-*response*		n	*Shape change*	
		Agonist (pEC_{50})	Antagonist (pIC_{50})		Agonist (pEC_{50})	Antagonist (pIC_{50})
5-HT	14	7.16 ± 0.24		10	7.08 ± 0.05	
Psilocin	3	6.30 ± 0.03		4	6.75 ± 0.10	
Tryptamine	3	5.40 ± 0.03		4	5.85 ± 0.08	
Quipazine	3	5.10 ± 0.01		4	5.67 ± 0.40*	
Spiroperidol	2		9.70;9.10	3		8.66 ± 0.06
Methiothepin	3		8.92 ± 0.12	3		8.39 ± 0.02
Ketanserin	7		7.29 ± 0.15	5		8.19 ± 0.10
D-Butaclamol	4		7.40 ± 0.02	4		7.77 ± 0.07
Cinanserin	3		7.22 ± 0.02	3		7.32 ± 0.09
L-Butaclamol	2		6.00;5.30	3		5.40 ± 0.05
Desimipramine	2		<4.00	3		5.89 ± 0.09
Yohimbine	2		<4.00	2		<4.50

pEC_{50} is defined as the negative logarithm of the molar concentration of drugs causing a half maximal rise of $[Ca^{2+}]_i$ or a half maximal shape change reaction. pIC_{50} is defined as the negative logarithm of the molar concentration of drugs causing a 50% inhibition of the maximal rise of $[Ca^{2+}]_i$ or of the maximal shape change induced by 10^{-6} M 5-hydroxytryptamine (5-HT). The figures are means with s.e. The values of the shape change experiments, except those for ketanserin, were in general taken from Laubscher & Pletscher (1979). *Value in rabbits (Graf & Pletscher, 1979).

Discussion

In the present experiments the rise of $[Ca^{2+}]_i$ in response to 5-HT was considerably faster than previously reported (Affolter *et al.*, 1984), its velocity now being of the same order as that of the shape change reaction. This indicates that the earlier results were influenced by an artifact connected with the relatively high intracellular levels of quin-2. The elevation of $[Ca^{2+}]_i$ was probably still retarded due to dampening by the recording system, but this would also delay the shape change reaction which occurs in a

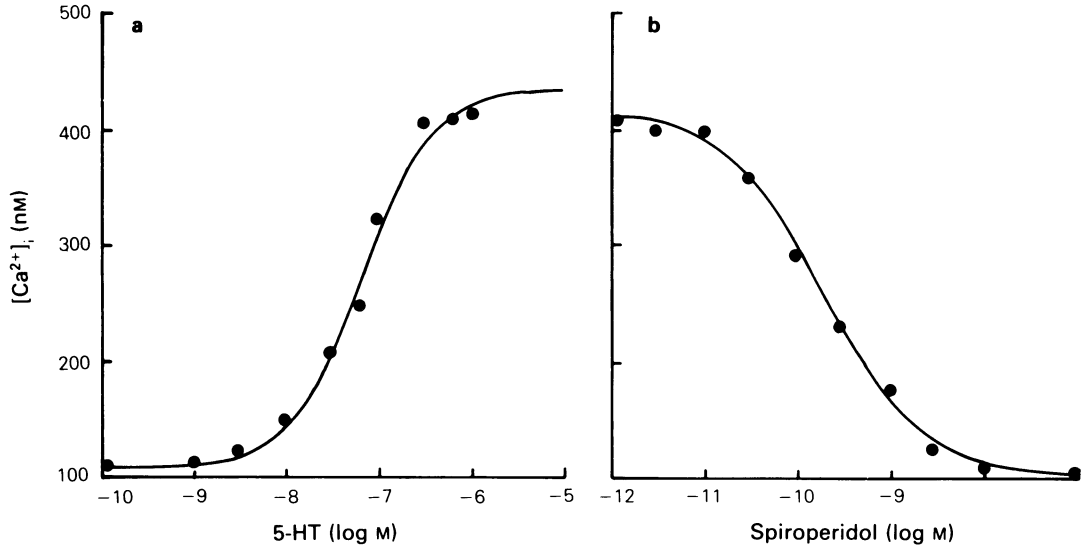

Figure 4 (a) Effect of various concentrations of 5-hydroxytryptamine (5-HT) on $[Ca^{2+}]_i$ of platelets incubated in a medium containing $CaCl_2$ 1 mM. (b) Counteraction by various concentrations of spiroperidol of the $[Ca^{2+}]_i$-rise induced by 5-HT 10^{-6} M. $[Ca^{2+}]_i$ was determined 40 s after addition of 5-HT. Typical experiments.

few seconds (Born *et al.*, 1978). In addition, the presence of even relatively small amounts of intracellular quin-2 is likely to cause a delay of the $[Ca^{2+}]_i$-rise (Tsien, 1981). Nevertheless, our present findings support the hypothesis proposed earlier that $[Ca^{2+}]_i$ has a mediator role in the 5-HT-induced shape change reaction.

Our experiments with buffers virtually devoid of Ca^{2+} (free extracellular Ca^{2+} below 10^{-11} M) also show that 5-HT caused a considerable mobilisation of intracellular calcium ($[Ca^{2+}]_i$ mobil 9.5×10^{-5} M). This conclusion is confirmed by the findings with TMB-8. TMB-8, which is thought to inhibit the liberation of calcium from internal stores (Malagodi & Chiou, 1974), counteracted the 5-HT-induced rise of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. The enhanced 5-HT-induced increase of Ca^{2+} in the presence of the extracellular cation was probably due to influx from the medium.

Release of intracellular Ca^{2+} by 5-HT explains why the amine still induces a shape change reaction in media containing EDTA. The slightly enhanced potency of 5-HT in the absence of EDTA may be the consequence of an influx of extracellular Ca^{2+} into the platelets.

Finally, the present findings with 5-HT-agonists and -antagonists support the previously expressed view (which was based on experiments with ketanserin; Affolter *et al.*, 1984) that the 5-HT-induced $[Ca^{2+}]_i$-rise, like the 5-HT-induced shape change reaction, is a consequence of stimulation of $5\text{-}HT_2$-receptors. In fact, several compounds known to act on 5-HT-receptors also affected $[Ca^{2+}]_i$, whereas DMI and yohimbine were ineffective (Table 1). Furthermore, there was stereospecificity. In addition, the 5-HT-antagonists were more potent than the agonists which is typical for drugs acting on $5\text{-}HT_2$-receptors (Peroutka & Snyder, 1979). Finally, the order of potency of the compounds in affecting $[Ca^{2+}]_i$ was similar to that in the shape change reaction.

In conclusion, the present experiments provide evidence for the hypothesis that stimulation of $5\text{-}HT_2$-receptors of platelets causes a rapid release of calcium from intracellular stores and that the subsequent increase of $[Ca^{2+}]_i$ mediates the 5-HT-induced shape change reaction, probably by activation of the contractile system.

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