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Action of 5-hydroxytryptamine in facilitating N-methyl-D-aspartate depolarization of cortical neurones mimicked by calcimycin, cyclopiazonic acid and thapsigargin

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1 The ability of calcimycin, cyclopiazonic acid and thapsigargin to facilitate the N-methyl-D-aspartate (NMDA)-mediated depolarization of cortical projection neurones was investigated by use of grease-gap recording and the results compared with the facilitation that results from activation of 5-hydroxytryptamine$_{2A}$ receptors.

2 Calcimycin (0.25 to 3 μM), cyclopiazonic acid (5 to 30 μM), and thapsigargin (10 to 300 nM) reversibly facilitated the NMDA (50 μM)-induced depolarization in the presence of tetrodotoxin. The concentration-response relationships were bell-shaped with a mean enhancement of 550% for calcimycin (1 μM) and approximately 400% for cyclopiazonic acid (20 μM) and thapsigargin (100 nM). At the highest concentration of each agent tested, no facilitation was observed.

3 Chlorpromazine (1 μM) partially restored a facilitation at 3 μM calcimycin and 300 nM thapsigargin. *Myo*-inositol (10 mM) and 100 nM staurosporine were both ineffective in this regard.

4 The depolarization elicited by 10 μM quisqualate or 5 μM kainate was not facilitated by 10 μM cyclopiazonic acid.

5 Calcimycin (0.5 μM), cyclopiazonic acid (20 μM), and thapsigargin (100 nM) elicited a significant facilitation in the presence of an antagonist cocktail consisting of D,L-2-amino-3-phosphonopropionic acid, prazosin, ritanserin, and scopolamine, although the magnitude of the facilitation was reduced.

6 Facilitation of the NMDA depolarization elicited by both 30 μM 5-hydroxytryptamine and 10 μM phenylephrine was eliminated in nominally Mg^{2+}-free medium. In contrast, the facilitation induced by 0.5 μM calcimycin remained intact.

7 *Bis*-(*o*-aminophenoxy)-ethane-N,N,N,N, tetraacetic acid aminoethoxy (50 μM) or perfusion with nominally Ca^{2+}-free medium eliminated facilitation of the NMDA depolarization induced by 30 μM 5-hydroxytryptamine and 100 nM thapsigargin.

8 The facilitation induced by both 30 μM 5-hydroxytryptamine and 1 μM calcimycin was reduced in a concentration-dependent manner by nifedipine (1 to 10 μM).

9 Calcimycin, cyclopiazonic acid and thapsigargin facilitate the NMDA depolarization in a manner which closely mimics the facilitation induced by 5-hydroxytryptamine. It is concluded that enhancement of the NMDA depolarization at cortical projection neurones results from an elevation of Ca^{2+} in the cytosol and that several sources of Ca^{2+} contribute to the facilitation.

Keywords: Calcimycin; cyclopiazonic acid; thapsigargin; 5-hydroxytryptamine; N-methyl-D-aspartate; cerebral cortex

Introduction

5-Hydroxytryptamine (5-HT) selectively facilitates the depolarization of rat cortical neurones mediated by N-methyl-D-aspartate (NMDA) receptors (Reynolds *et al.*, 1988; Mally *et al.*, 1991; Neuman & Rahman, 1992; Rahman & Neuman, 1993a). The pharmacological profile of the 5-HT-induced facilitation is consistent with activation of the 5-HT$_{2A}$ receptor subtype, but not the closely related 5-HT$_{2C}$ receptor subtype (Neuman & Rahman, 1992; Rahman & Neuman, 1993a). Thus, the 5-HT-induced facilitation is antagonized by ritanserin and spiperone with IC_{50} values of 0.9 nM and 0.4 nM respectively. The facilitation is mimicked by (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, a mixed 5-HT$_{2A}$ and 5-HT$_{2C}$ agonist (Hoyer *et al.*, 1994), but not by N-(3-trifluoromethylphenyl)piperazine, a mixed 5-HT$_{1B}$ and 5-HT$_{2C}$ receptor agonist/5-HT$_{2A}$ receptor antagonist (Sills *et al.*, 1984; Conn & Sanders-Bush, 1987). *In situ* hybridization reveals that mRNA for the 5-HT$_{2A}$ receptor subtype is present in rat layer V pyramidal neurones (Rahman *et al.*, 1995), corroborating the electrophysiological (Davies *et al.*, 1987; Reynolds *et al.*, 1988; Araneda & Andrade, 1991; Rahman & Neuman, 1993a; Tanaka & North, 1993; Rahman *et al.*, 1995) and cross-desensitization evidence (Rahman & Neuman, 1993b,c) supporting the hypothesis that 5-HT acts directly through 5-HT$_{2A}$ receptors located on these neurones.

Intracellular recordings reveal that several K^+ conductances, including a resting K^+ conductance and the slow after hyperpolarization, are reduced following activation of 5-HT$_{2A}$ receptors on cortical neurones (Davies *et al.*, 1987; Araneda & Andrade, 1991; Tanaka & North, 1993). However, the 5-HT-induced facilitation appears to be unrelated to a reduction of a K^+ conductance as the facilitation persists in the presence of K^+ channel blockers and is not occluded by agents that mimic the 5-HT$_{2A}$ receptor-mediated depolarization or block the slow afterhyperpolarization (Reynolds *et al.*, 1988; Rahman & Neuman, 1993a,b).

The 5-HT$_{2A}$ receptor is a member of the guanine nucleotide binding protein (G-protein) coupled receptor superfamily (Hoyer *et al.*, 1994). In common with a number of other members of this family the receptor couples to phospholipase C (PLC) (Berridge, 1987; Chuang, 1989). At cortical neurones,

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facilitation of the NMDA depolarization elicited by 5-HT probably results as a consequence of activating PLC. Thus, other receptors which couple positively to PLC including α_1-adrenoceptors (Berridge, 1993), metabotropic glutamate receptors (mGluR) (Pin & Duvoisin, 1995) and muscarinic acetylcholine receptors (AChR) (Berridge, 1993) also facilitate the NMDA-induced depolarization of cortical neurones (Rahman & Neuman, 1993a; 1996). Moreover, increasing the concentration of substrate for PLC by perfusing *myo*-inositol (Fain & Berridge, 1979; Berridge, 1987), potentiates the 5-HT-induced facilitation in a concentration-dependent manner, reduces homologous desensitization of the 5-HT_{2A} receptor-mediated facilitation, and eliminates 5-HT-induced heterologous desensitization of the facilitation mediated by α_1-adrenoceptors and muscarinic AChR (Rahman & Neuman, 1993c).

PLC hydrolyses inositol 1,4 biphosphate resulting in the formation of diacylglycerol and inositol 1,4,5 trisphosphate (IP_3) (Berridge, 1987). Diacylglycerol activates protein kinase C (Berridge, 1987). Introduction of the catalytic subunit of protein kinase C into hippocampal neurones facilitates NMDA-evoked currents (Aniksztejn *et al.*, 1991; 1992). Responses of NMDA receptors expressed in *Xenopus* oocytes are also enhanced following the stimulation of protein kinase C with phorbol esters (Kelso *et al.*, 1992). On the other hand, stimulation of protein kinase C does not facilitate the NMDA depolarization at cortical neurones and inhibition of protein kinase C potentiates the facilitation induced by 5-HT (Rahman & Neuman, 1993b; Rahman *et al.*, 1995). Moreover, stimulation and inhibition of protein kinase C does not alter the facilitation elicited by activating mGluR at cortical neurones (Rahman & Neuman, 1996).

IP_3 mobilizes Ca^{2+} following binding to IP_3 receptors located on intracellular Ca^{2+} storage sites (Berridge, 1987; 1993; Chuang, 1989; Henzi & MacDermott, 1992; Pozzan *et al.*, 1994). The elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) that takes place following activation of IP_3 receptors has been proposed to underlie facilitation of the NMDA depolarization induced by the activation of mGluR, muscarinic AChR, and 5-HT_{2A} receptors (Markram & Segal, 1991; 1992; Rahman & Neuman, 1993a; 1996; Kong & Neuman, 1995). If such a mechanism is responsible for the facilitation, then elevating $[Ca^{2+}]_i$ independently of IP_3 production should also facilitate the NMDA depolarization. To test this hypothesis we employed calcimycin, a Ca^{2+} ionophore (Pressman, 1976), cyclopiazonic acid, an indole tetramic acid metabolite of *Aspergillus* and *Penicillium* (Seidler *et al.*, 1989), and thapsigargin, a naturally occurring sesquiterpene lactone (Thastrup *et al.*, 1990). These structurally unrelated agents share in common an ability to elevate $[Ca^{2+}]_i$ without activation of cell surface receptors that couple to PLC. Cyclopiazonic acid and thapsigargin increase $[Ca^{2+}]_i$ through the selective inhibition of smooth endoplasmic reticulum Ca^{2+}-ATPase (Thastrup *et al.*, 1990; Verma *et al.*, 1990; Mason *et al.*, 1991), whereas calcimycin forms divalent selective cationic ionophores (Pressman, 1976; Pozzan *et al.*, 1994). Part of this material has been communicated in preliminary form (Rahman & Neuman, 1993d; Neuman & Rahman, 1993; 1994; 1996).

Methods

Cortical slice preparation and recording

Male 100–300 g Sprague-Dawley rats (Charles River, Montreal, Quebec) were used; wedges from the sensorimotor cortex were prepared from 500 μM thick coronal slices for recording as described (Harrison & Simmonds, 1985; Rahman & Neuman, 1993a). Following at least 1 h of recovery, a wedge of cortex (1.5 mm at the pial surface and 1 mm at the corpus callosum) was mounted in a two compartment grease-gap recording apparatus (Harrison & Simmonds, 1985). The bath (1.5 ml volume) was perfused at 2 ml min^{-1} with artificial cerebrospinal fluid (ACSF) at room temperature (20 to 22°C). Excitatory amino acid-induced depolarization of the cell bodies was recorded with respect to the corpus callosum using Ag/AgCl electrodes (embedded in 3% agar in 1 M NaCl) connected to a differential amplifier (d.c. to 0.1 Hz). ACSF had the following composition (mM): NaCl 126, KCl 3.5, $CaCl_2$ 2, $MgCl_2$ 1.3, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, and tetrodotoxin 0.0001 or 0.0003. The ACSF was aerated with 95% O_2/5% CO_2 and had a pH of 7.4.

Drugs were dissolved in ACSF and applied to the cell body containing compartment. NMDA was applied for 2 min every 20 min at a concentration of 50 μM, which previously had been shown to produce the largest facilitation in the presence of 5-HT (Rahman & Neuman, 1993a). Phenylephrine and 5-HT were co-administered with NMDA for 2 min. Co-application of calcimycin, cyclopiazonic acid or thapsigargin with NMDA for 2 min resulted in responses with considerable variation. However, a 3 min application starting 1 min prior to NMDA and continuing with NMDA resulted in a consistent response and this protocol was therefore adopted. *Bis*-(*o*-aminophenoxy)-ethane-N,N,N,N, tetraacetic acid aminoethoxy (BAPTA-AM), calcimycin, cyclopiazonic acid, staurosporine, and thapsigargin were dissolved in dimethylsulphoxide and diluted in ACSF. Equivalent dilutions of dimethylsulphoxide alone did not alter the NMDA depolarization. Nifedipine was dissolved in ethanol and diluted.

Data analysis

Amplitude of the depolarization was converted to percentage of control [(treatment/control) × 100] and the geometric mean determined (Gaddum, 1945). As appropriate, data were analysed by paired *t* tests or by one-way analysis of variance (Instat, GraphPad Software) followed by the Bonferroni test if the *F* value was significant. Data are presented as the antilog of the geometric mean ± s.e.mean. To avoid concern with desensitization (Rahman & Neuman, 1993b), comparisons were usually made between control and treatment conditions by use of separate sets of wedges for each condition.

Drugs and chemicals

D,L-2-Amino-3-phosphonopropionic acid (D,L-AP3), BAPTA-AM and thapsigargin were obtained from Calbiochem. Chlorpromazine HCl, prazosin HCl, and ritanserin were gifts from Rhone-Poulenc Pharma, Pfizer, and Janssen, respectively. Calcimycin, cyclopiazonic acid, kainic acid, *myo*-inositol, N-methyl-D-aspartate, nifedipine, phenylephrine, quisqualate, scopolamine HBr, 5-hydroxytryptamine bimaleate salt, staurosporine and tetrodotoxin were obtained from Sigma. Drug concentrations were calculated as the salt. Stock solutions were kept frozen until use.

Results

Thapsigargin, cyclopiazonic acid and calcimycin enhance the NMDA depolarization

NMDA (50 μM) depolarized cortical neurones and, in keeping with previous observations (Nedergaard *et al.*, 1987; Reynolds *et al.*, 1988; Mally *et al.*, 1991; Rahman & Neuman, 1993a), 30 μM 5-HT reversibly facilitated the depolarization (Figure 1). Application of 5-HT in the absence of NMDA does not alter the potential in grease-gap recording (Mally *et al.*, 1991; Rahman & Neuman, 1993a). Thapsigargin (100 nM) alone did not induce a depolarization (data not shown); however, it reversibly increased the amplitude of the NMDA depolarization (Figure 1).

Calcimycin and cyclopiazonic acid also facilitated the NMDA-induced depolarization without eliciting a depolarization when perfused alone. The concentration-response relationships for the calcimycin; cyclopiazonic acid- and thapsigargin-induced facilitation are shown in Figure 2. The

curves are remarkably similar except for differences in potency and the larger enhancement elicited by calcimycin. All three agents facilitated the NMDA depolarization in a concentration-dependent manner up to a peak, followed by a rapid decline in the degree of facilitation with a further increase of concentration.

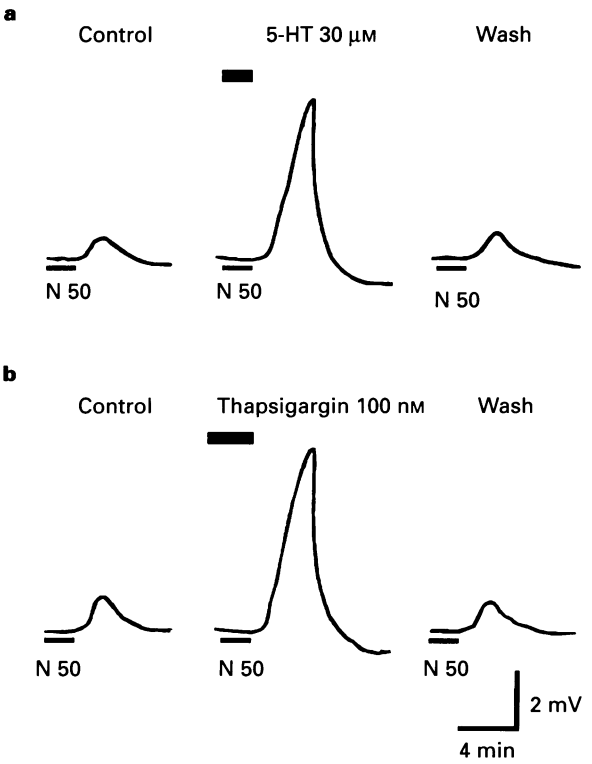

Figure 1 Facilitation of NMDA depolarization by 5-hydroxytryptamine (5-HT) and thapsigargin. NMDA (N, 50 μM) depolarizes cortical neurones with respect to the corpus callosum (Control). Administration of 5-HT (a) and thapsigargin (b) facilitates the NMDA response. Recovery 20 min later (Wash). Data from separate wedges. Traces were digitized from chart recordings by use of a hand scanner.

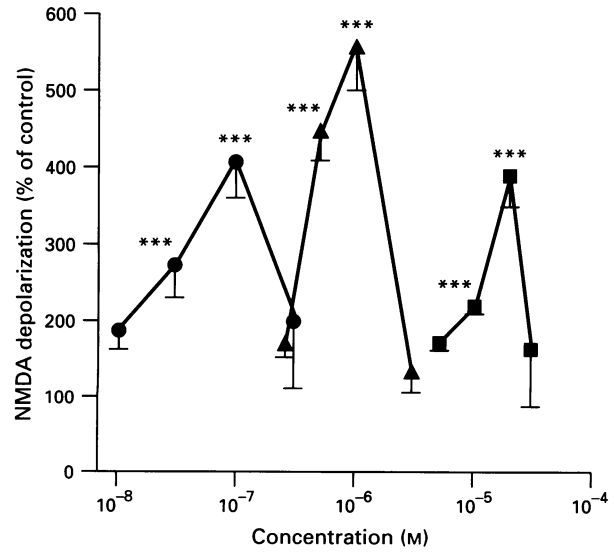

Figure 2 The concentration-response relationship for the facilitation elicited by calcimycin (▲), cyclopiazonic acid (■) and, thapsigargin (●). NMDA (N) concentration was 50 μM; 3 to 8 wedges were tested at each concentration. ***$P<0.001$; NMDA control vs. treatment.

Since calcimycin, cyclopiazonic acid, and thapsigargin continue to elevate $[Ca^{2+}]_i$ at concentrations larger than those employed in the present study, the observed decline of the facilitation might represent desensitization triggered by Ca^{2+}. Calmodulin is activated by Ca^{2+} (Erondu & Kennedy, 1985) and one form of desensitization observed with the 5-HT-induced facilitation is sensitive to antagonists of calmodulin, calmidazolium and chlorpromazine (Rahman & Neuman, 1993b). Chlorpromazine (1 μM) reduced the decline of the facilitation when perfused before calcimycin and thapsigargin (Table 1). Activation of protein kinase C also contributes to desensitization of the 5-HT-induced facilitation (Rahman & Neuman, 1993b). Staurosporine (10 nM), a nonspecific inhibitor of protein kinase C (Ruegg & Burgess, 1989) did not prevent loss of the facilitation (Table 1). Depletion of substrate for PLC does not appear to underlie loss of the calcimycin facilitation since 10 mM *myo*-inositol, which reduces 5-HT-induced desensitization (Rahman & Neuman, 1993c), also failed to restore the facilitation (Table 1).

The depolarization induced by NMDA is enhanced by 5-HT, but not the depolarization induced by quisqualate or kainate (Reynolds *et al.*, 1988; Rahman & Neuman, 1993a). No facilitation was observed with 10 μM cyclopiazonic acid when 10 μM quisqualate or 5 μM kainate were substituted for NMDA (103±4%, $n=4$ and 92±4%, $n=3$, respectively).

Indirect effects related to transmitter release

In cortical wedges, release of acetylcholine and noradrenaline is sufficient to facilitate the NMDA-induced depolarization (Rahman & Neuman, 1993a). Part of this release may be dependent on the NMDA receptor-mediated depolarization of nerve terminals and therefore insensitive to tetrodotoxin (Wang *et al.*, 1992). Thus, despite the presence of tetrodotoxin, calcimycin, cyclopiazonic acid and thapsigargin might facilitate the NMDA-induced depolarization indirectly through enhanced release of neurotransmitters which activate receptors that couple to PLC. To test this possibility, calcimycin, cyclopiazonic acid and thapsigargin were applied in the presence of a 'cocktail' which consisted of D,L-AP3 (50 μM), prazosin (1 μM), scopolamine (10 nM), and ritanserin (10 nM). These antagonists reduce the facilitation resulting from activation of mGluR, α_1-adrenoceptors, muscarinic AChR, and 5-HT_{2A} receptors, respectively (Rahman & Neuman, 1993a; 1996). Perfusion of the antagonist cocktail reduced the amplitude of NMDA depolarization (Table 2) as expected (Rahman & Neuman, 1993a). However, the magnitude of the facilitation induced by calcimycin, cyclopiazonic acid and thapsigargin remained statistically significant, although the extent of the facilitation was reduced in comparison to the control (Table 2). These results suggest (i) enhanced transmitter release or (ii) additivity/synergism between calcimycin, cyclopiazonic acid,

Table 1 Chlorpromazine, but not *myo*-inositol or staurosporine, partially restores facilitation of the NMDA response at elevated concentrations of calcimycin and thapsigargin

Treatment		*NMDA depolarization* (% of control)
Calcimycin 1 μM		554±55 ($n=5$)
Calcimycin 3 μM		132±24 ($n=4$)
Calcimycin 3 μM	+chlorpromazine 1 μM	236±14 ($n=4$)*
Calcimycin 3 μM	+*myo*-inositol 10 mM	133±17 ($n=4$)
Calcimycin 3 μM	+staurosporine 10 nM	142±22 ($n=4$)
Thapsigargin 0.1 μM		407±46 ($n=5$)
Thapsigargin 0.3 μM		200±88 ($n=3$)
Thapsigargin 0.3 μM	+chlorpromazine 1 μM	300±62 ($n=3$)*

*$P<0.05$, treatment vs. calcimycin 3 μM or thapsigargin 0.3 μM

thapsigargin and the action of one or more transmitters acting at the postsynaptic neurones.

To reduce the possibility of transmitter involvement another strategy was required. In nominally Mg^{2+}-free ACSF the muscarinic AChR-induced depolarization of cortical neurones is eliminated (El-Beheiry & Puil, 1990). This loss probably reflects the requirement for cytosolic Mg^{2+} in the effective operation of G-protein coupled receptors (Birnbaumer *et al.*, 1990; El-Beheiry & Puil, 1990; Litosch, 1991). To determine whether other G-protein coupled receptors were sensitive to Mg^{2+}-free conditions, the ability of 5-HT and phenylephrine to evoke a facilitation was examined. As expected (Nowak *et al.*, 1984), perfusing nominally Mg^{2+}-free ACSF enhanced the depolarization evoked by 50 μM NMDA (data not shown). To maintain approximately the same level of depolarization in Mg^{2+}-free ACSF, the concentration of NMDA was reduced to 10 μM. When applied in nominally Mg^{2+}-free ACSF, 30 μM 5-HT failed to facilitate the depolarization evoked by 10 μM NMDA (Figure 3; Table 3). Phenylephrine (10 μM) (Rahman & Neuman, 1993b) also failed to elicit a facilitation (Table 3). In marked contrast, 1 μM calcimycin induced a facilitation in nominally Mg^{2+}-free ACSF (Figure 3; Table 3).

Table 2 Blockade of α_1-adrenoceptors, muscarinic AChR, mGluR, and 5-HT_{2A} receptors reduced but did not eliminate, the facilitation induced by calcimycin, cyclopiazonic acid, and thapsigargin

Treatment	*NMDA depolarization* (% of control)
NMDA 50 μM	100
+antagonist cocktail	70±4 (n=15)***
Calcimycin 0.5 μM	444±35 (n=5)***
+antagonist cocktail	203±19 (n=5)***
Cyclopiazonic acid 20 μM	389±41 (n=6)***
+antagonist cocktail	149±7 (n=5)***
Thapsigargin 100 nM	407±46 (n=6)***
+antagonist cocktail	140±16 (n=5)**

The antagonist cocktail consisted of D,L-AP3 (50 μM), prazosin (1 μM), ritanserin (10 nM), scopolamine (10 nM), and tetrodotoxin (0.3 μM). **P<0.01, ***P<0.001, treatment vs. NMDA or NMDA plus antagonist as appropriate

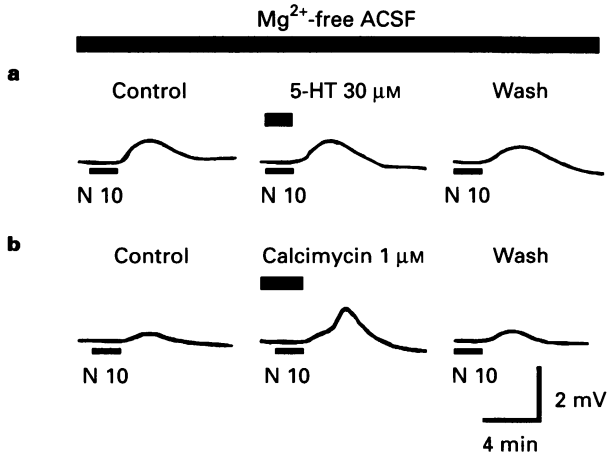

Figure 3 Effect of Mg^{2+}-free ACSF on the facilitation elicited by 5-HT and calcimycin. The concentration of NMDA (N) was reduced to 10 μM as the response to 50 μM NMDA in Mg^{2+}-free ACSF was substantially increased. (a) The 5-HT facilitation was absent in nominally Mg^{2+}-free ACSF. (b) The calcimycin facilitation persists under the same conditions. Data from two wedges.

Ca^{2+}-dependence of the facilitation

In rat cortical slices, phosphoinositide hydrolysis induced by both 5-HT and noradrenaline is significantly reduced in nominally Ca^{2+}-free ACSF (Kendall & Nahorski, 1984). The phenylephrine-evoked facilitation is also eliminated in nominally Ca^{2+}-free ACSF (Rahman & Neuman, 1996). In a similar vein, the application of 30 μM 5-HT in nominally Ca^{2+}-free ACSF failed to facilitate the NMDA response (Figure 4; Table 4). BAPTA-AM (50 μM), which is cell-permeable and chelates only intracellular Ca^{2+} (Niesen *et al.*, 1991), reduced the amplitude of the NMDA depolarization (Table 4) and also eliminated the 5-HT-induced facilitation (Figure 4; Table 4). Application of 100 nM thapsigargin in nominally Ca^{2+}-free ACSF or in the presence of BAPTA-AM yielded results similar to those obtained with 5-HT, i.e. in both cases the facilitation was eliminated (Figure 4; Table 4).

Nifedipine blocks the facilitation

Both receptor-operated channels and voltage-dependent Ca^{2+} channels contribute to Ca^{2+} entry at cortical pyramidal neurones (Markram & Sakmann, 1994; Markram *et al.*, 1995). Voltage-dependent L-type Ca^{2+} channels on these neurones

Table 3 Perfusion of nominally Mg^{2+}-free ACSF eliminates the 5-HT-and phenylephrine-induced facilitation, whereas the calcimycin facilitation is preserved

Treatment	*NMDA depolarization* (% of control)
5-HT (30 μM)	100±1 (n=6)
Phenylephrine (10 μM)	99±9 (n=6)
Calcimycin (1 μM)	318±43 (n=4)**

The concentration of NMDA employed was 10 μM. **P<0.01, treatment vs. control

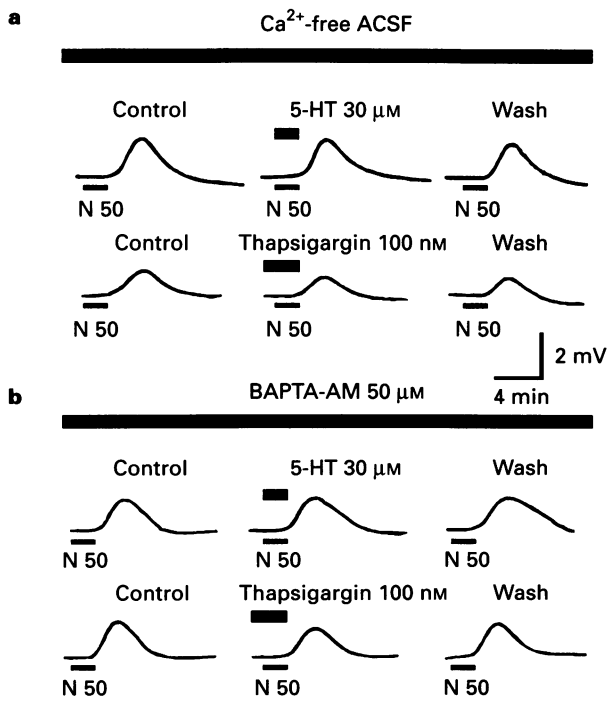

Figure 4 Thapsigargin and 5-HT fail to induce facilitation during perfusion with (a) nominally Ca^{2+}-free ACSF or (b) BAPTA-AM. Data from four wedges.

are blocked by nifedipine (Sayer *et al.*, 1992). Application of nifedipine reduced both the 5-HT (30 μM) and the calcimycin (1 μM)-induced facilitation in a concentration-dependent manner (Figure 5).

Discussion

The present observations demonstrate that the facilitation of the NMDA response induced by calcimycin, cyclopiazonic acid and thapsigargin is similar to that induced by 5-HT, except that the 5-HT facilitation requires Mg^{2+}. Thus, the facilitation: (1) is selective for the depolarization induced by NMDA receptor activation; (2) is absent in nominally Ca^{2+}-free ACSF; (3) is eliminated in cortical wedges perfused with BAPTA-AM and (4) undergoes a concentration-dependent blockade by nifedipine. This correspondence suggests that a common mechanism underlies the facilitation induced by both the 5-HT_{2A} cell surface receptor coupled to PLC and agents which promote a rise in $[Ca^{2+}]_i$.

The IP_3-sensitive Ca^{2+} pool, located in the smooth endoplasmic reticulum, is maintained, in the face of a constant leak of Ca^{2+}, by a Ca^{2+}-ATPase. Cyclopiazonic acid and thapsigargin inhibit this Ca^{2+}-ATPase leading to the loss of Ca^{2+} from the storage site and a concomitant rise in $[Ca^{2+}]_i$ (Thastrup *et al.*, 1990; Verma *et al.*, 1990; Mason *et al.*, 1991). The concentrations of cyclopiazonic acid and thapsigargin which elicit a facilitation are similar to those necessary to increase $[Ca^{2+}]_i$ (Mason *et al.*, 1991). Moreover, the magnitude of the facilitation induced by cyclopiazonic acid and thapsigargin is comparable, consistent with a common mechanism of action. Cyclopiazonic acid and thapsigargin differ in their action from neurotransmitters and hormones that couple to PLC in that the increase in $[Ca^{2+}]_i$ they evoke is not accompanied by a rise in IP_3 (Jackson *et al.*, 1988; Thastrup *et al.*, 1990).

Table 4 The facilitation induced by 5-HT and thapsigargin is dependent on external Ca^{2+} and is eliminated when internal Ca^{2+} is buffered

Treatment	*NMDA depolarization* (% of control)
Nominally Ca^{2+}-free ACSF	
5-HT (30 μM)	99±6 (n=5)
Thapsigargin (100 nM)	109±5 (n=5)
BAPTA-AM (50 μM)	
NMDA (50 μM)	61±6 (n=5)**
5-HT (30 μM)	110±7 (n=5)
Thapsigargin (100 nM)	106±5 (n=4)

**P=0.0018, treatment vs. NMDA control

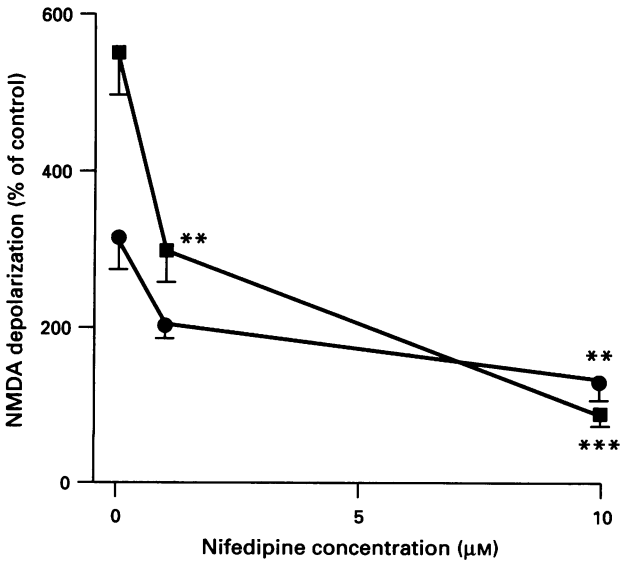

Figure 5 Nifedipine reduces the facilitation elicited by 1 μM calcimycin (■) and 30 μM 5-HT (●) in a concentration-dependent manner. Five to 6 wedges were used at each concentration. **P<0.01; ***P<0.001; nifedipine plus agonist vs. nifedipine control.

Calcimycin evokes a greater maximum response with respect to the facilitation than does cyclopiazonic acid or thapsigargin. This divergence may reflect calcimycin's mode of action, i.e. forming divalent selective cation ionophores that allow Ca^{2+} to enter the cytosol from both the extracellular space and from cellular storage sites (Pressman, 1976; Pozzan *et al.*, 1994). Calcimycin may also raise $[Ca^{2+}]_i$ indirectly through stimulation of phosphoinositide hydrolysis (Fisher & Agranoff, 1981; Brammer *et al.*, 1988; Brammer & Weaver, 1989), although it exhibits a pattern of inositol polyphosphate formation unlike that which results from activation of cell surface receptors that couple to PLC (Baird & Nahorski, 1990).

Loss of the facilitation at elevated concentrations of calcimycin, cyclopiazonic acid and thapsigargin is reminiscent of the desensitization exhibited by the 5-HT facilitation (Rahman & Neuman, 1993b). In the case of 5-HT, the desensitization is related to depletion of substrate, activation of protein kinase C, receptor internalization, and activation of calmodulin (Rahman & Neuman, 1993b, c).

Myo-inositol and staurosporine both failed to reduce the decline observed with 3 μM calcimycin, suggesting that the decrease does not result from the exhaustion of substrate for PLC (Rahman & Neuman, 1993c) or activation of protein kinase C (Rahman & Neuman, 1993b). By contrast, in the presence of chlorpromazine there remained a significant facilitation in response to 3 μM calcimycin and 300 nM thapsigargin. Chlorpromazine and calmidazolium also reduce desensitization of the 5-HT-induced facilitation (Rahman & Neuman, 1993b), which is consistent with a common postsynaptic mechanism underlying loss of the facilitation observed with calcimycin and thapsigargin on the one hand and 5-HT on the other. Like other lipophilic molecules, chlorpromazine can antagonize calmodulin (Weiss *et al.*, 1982). Calcimycin, cyclopiazonic acid, and thapsigargin as well as activation of 5-HT_{2A} receptors could stimulate calmodulin by raising $[Ca^{2+}]_i$ (Erondu & Kennedy, 1985) and this might account for loss of the facilitation.

Similarities in the facilitation elicited by 5-HT on the one hand and agents that promote Ca^{2+} entry/release without activating cell surface receptors on the other might simply reflect the ability of the latter to enhance the release of neurotransmitters which activate receptors on projection neurones that couple to PLC (cf. Markram & Segal, 1991). The depolarization of cortical neurones evoked by NMDA is significantly reduced by prazosin and scopolamine, which suggests that the release of endogenous acetylcholine and noradrenaline facilitates the NMDA response (Rahman & Neuman, 1993a). Indeed, the observation that the antagonist cocktail dramatically reduced the facilitation evoked by calcimycin, cyclopiazonic acid and thapsigargin implies that one or more neurotransmitters make a substantial contribution to the facilitation. The facilitation that remains in the presence of the antagonist cocktail might result from incomplete blockade by one or more of the antagonists or the action of a neurotransmitter for which an antagonist was not present. Blockade of L-type Ca^{2+}-channels with nifedipine, reduction in extracellular Ca^{2+}, and buffering $[Ca^{2+}]_i$ with BAPTA-AM all decrease evoked transmitter release (Sanchez-Prieto *et al.*, 1987; Niesen *et al.*, 1991). Thus, the effectiveness of these agents and treatment in eliminating the facilitation induced by calcimycin, cyclopiazonic acid, and thapsigargin could be taken as support

for a strictly presynaptic mechanism of action. Although such an interpretation cannot be entirely excluded at this time, it does appear improbable.

The facilitation induced by both 5-HT and phenylephrine, but not that induced by calcimycin, is eliminated in nominally Mg^{2+}-free ACSF. Activation of G-protein complexes requires Mg^{2+} (Birnbaumer *et al.*, 1990; Litosch, 1991); removal of extracellular Mg^{2+}, which presumably lowers the concentration of Mg^{2+} in the cytosol (Heinonen & Åkerman, 1986), is sufficient to depress the muscarinic AChR-evoked depolarization of neocortical neurones (El-Beheiry & Puil, 1990). Thus, it is plausible that the Mg^{2+}-dependence of the facilitation induced by G-protein coupled receptors reflects the regulatory role of Mg^{2+} in signal transduction associated with these receptors, rather than loss of voltage-dependence at the NMDA receptor-gated ion channel (Chen & Huang, 1992; see also Nedergaard *et al.*, 1987). Such a mechanism would account for the insensitivity of the calcimycin facilitation to the reduction in Mg^{2+}. Moreover, the failure of nominally Mg^{2+}-free ACSF to eliminate the calcimycin-induced facilitation is incompatible with release of a G-protein coupled neurotransmitter being solely responsible for the facilitation. We therefore suggest that calcimycin, cyclopiazonic acid, and thapsigargin act, at least in part, at postsynaptic neurones to facilitate the NMDA response.

In the present investigation, the omission of Ca^{2+} from the ACSF eliminated the facilitation elicited by both 5-HT and thapsigargin. 5-HT-stimulated IP_3 production is reduced in Ca^{2+}-free ACSF (Kendall & Nahorski, 1984), which could account for loss of the facilitation. Consistent with this interpretation, α_1-adrenoceptor-stimulated IP_3 generation is reduced in nominally Ca^{2+}-free ACSF (Kendall & Nahorski, 1984) and facilitation of the NMDA response elicited by phenylephrine is eliminated (Rahman & Neuman, 1996). In contrast, IP_3 production following activation of muscarinic AChR and mGluR is far less sensitive to external Ca^{2+} (Kendall & Nahorski, 1984; Birrell & Marcoux, 1993; Challiss *et al.*, 1994) and the facilitation mediated by stimulating these receptors is not blocked during perfusion with nominally Ca^{2+}-free ACSF (Rahman & Neuman, 1996). Thapsigargin continues to elevate $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, although the magnitude of the peak is smaller and the sustained phase is absent (Mason *et al.*, 1991; Pozzan *et al.*, 1994). Thus, the absence of the thapsigargin facilitation in nominally Ca^{2+}-free ACSF suggests that a threshold level of $[Ca^{2+}]_i$ is required to facilitate the NMDA response. Multiple sources of Ca^{2+} including Ca^{2+} entry through voltage-dependent Ca^{2+} channels and the NMDA receptor ionophore (Markram & Sakmann, 1994; Markram *et al.*, 1995) as well as Ca^{2+} release from the ryanodine-sensitive Ca^{2+} pool (Rahman, Kong, Asgar, Giles & Neuman, unpublished observations) may well contribute to attaining this threshold.

One important source of Ca^{2+} for the facilitation appears to be Ca^{2+} entry through voltage-dependent L-type Ca^{2+} channels. These channels would be activated by the depolarization evoked by NMDA (see Markram & Sakmann, 1994). Nifedipine blocks both the 5-HT- and the calcimycin-mediated facilitation in a concentration-dependent manner. The concentration of nifedipine employed is in keeping with the concentration necessary to block L-type Ca^{2+} channels at pyramidal neurones (Sayer *et al.*, 1992), but is less than the concentration necessary to block Na^+/Ca^{2+} exchange or ATP-dependent Ca^{2+} uptake (Carvalho *et al.*, 1986). The facilitation induced by the mGluR agonist 1**S**, 3**R**-1-aminocyclopentane-1,3-dicarboxylic acid, which blocks nifedipine-sensitive Ca^{2+} channels (Sayer *et al.*, 1992), is insensitive to nifedipine (Rahman & Neuman, 1996) demonstrating that (i) nifedipine does not directly reduce the facilitation and (ii) the facilitation does not exhibit an absolute requirement for Ca^{2+} entry through L-type Ca^{2+} channels (Rahman & Neuman, 1996).

That an elevation of $[Ca^{2+}]_i$ is essential for the NMDA facilitation is revealed by our observations with BAPTA-AM. BAPTA-AM eliminates the NMDA facilitation independent of the agent used to induce the facilitation (see also Rahman & Neuman, 1996). In whole cell recordings from cortical neurones, buffering $[Ca^{2+}]_i$ to low nanomolar values with EGTA eliminates both muscarinic AChR and mGluR facilitation of the NMDA-evoked current (Kong & Neuman, unpublished observations). At hippocampal neurones, facilitation of the NMDA response induced by carbachol and calcimycin is also eliminated by the diffusion of BAPTA from an intracellular electrode (Markram & Segal, 1991; 1992).

In conclusion, calcimycin, cyclopiazonic acid, and thapsigargin facilitate the depolarization of cortical projection neurones induced by NMDA and this facilitation closely mimics that induced by 5-HT. An elevation of $[Ca^{2+}]_i$ is envisioned as the common mechanism underlying the facilitation. Since application of elevated Ca^{2+} to the cytosolic face of inside-out patches reveals a pronounced inactivation of NMDA-evoked currents (Vyklicky, 1993), a rise in $[Ca^{2+}]_i$ is presumed to regulate indirectly the NMDA receptor (Wang *et al.*, 1994; Wang & Salter, 1994; Lieberman & Mody, 1994) through activation of a Ca^{2+}-dependent protein.

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