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DISTINCT RECEPTORS, SECOND MESSENGERS AND CONDUCTANCES UNDERLYING THE DUAL RESPONSES TO SEROTONIN IN AN IDENTIFIED LEECH NEURONE

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Summary

1. Pressure-sensitive mechanosensory (P) neurones of the leech *Hirudo medicinalis* produce two responses to serotonin (5-HT): activation of a Cl^- conductance and of a non-selective monovalent cation conductance. The effects of channel blockers, the receptor pharmacology and the second-messenger dependence of these responses were studied in voltage-clamped P cells in culture. Antagonists were applied by superfusion and agonists by pressure ejection.

2. Zn^{2+} (100 μmol l^{-1}) and H^+ (pH 6.5 and lower) reversibly reduced the Cl^- conductance activated by 5-HT. The cation conductance was impermeant to calcium ions and was reduced by micromolar concentrations of the Na^+ channel inhibitors amiloride and 3,4-dichlorobenzamil.

3. High concentrations of antagonists or agonists of 5-HT_1 receptors and an antagonist of 5-HT_3 receptors had no effect on either response of P cells to 5-HT. Micromolar concentrations of ketanserin or cyproheptadine, which selectively antagonize 5-HT_2 receptors, reduced the cation but not the Cl^- conductance. From these results, the receptor underlying the cation conductance appears to be of the 5-HT_2 subtype, whereas the receptor activating the Cl^- conductance does not fit within the mammalian classification scheme.

4. Brief (<500 ms) application of membrane-permeant agonists of the second messenger cyclic AMP elicited a Cl^- conductance, whereas antagonists of cyclic-AMP-dependent protein kinase A reversibly suppressed the Cl^- conductance elicited by 5-HT and by cyclic AMP agonists. Compounds affecting other second messenger pathways were without effect on the Cl^- conductance. It therefore appears that the Cl^- conductance is activated by cyclic-AMP-dependent protein kinase A.

5. Cyclic nucleotide agonists and antagonists were without effect on the cation conductance. However, brief application of phorbol esters, which activate protein kinase C, elicited an amiloride-sensitive cation current. An inhibitor of protein

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kinase C reduced the cation conductance activated by 5-HT and by phorbol esters. Therefore, the cation conductance appears to depend on activation of protein kinase C.

6. We conclude that 5-HT activates two types of receptor coupled to separate ionic channels *via* different second messenger pathways in P cells. A receptor that is distinct from the mammalian subtypes activates Cl^- channels *via* cyclic-AMP-dependent protein kinase A. 5-HT_2 receptors appear to activate cation channels by means of protein kinase C.

Introduction

The displacement of 5-HT binding to receptors in mammalian neuronal preparations by a variety of agents has led to the classification of several major classes of binding sites. 5-HT_1 binding sites were first identified by their higher affinity for 5-HT than for spiperone, as opposed to 5-HT_2 sites which show the opposite affinity (Peroutka and Snyder, 1979). In recent years, compounds with greater (but incomplete) selectivity for these two sites have been identified or synthesized. These drugs have permitted the classification of 5-HT_1 binding sites into four subtypes (1A, 1B, 1C and 1D) and have been used to identify a third class of binding sites (5-HT_3) in the nervous system (Peroutka, 1988). Each of these binding sites corresponds to a 5-HT receptor that mediates physiological effects in the peripheral or central nervous systems (Bobker and Williams, 1990).

The receptors are differentially distributed (Peroutka, 1988) and many neurones in vertebrates (Nicoll, 1988) and in invertebrates (Gerschenfeld, 1973) show multiple responses to 5-HT which depend on the pattern of synaptic inputs. In many neurones, 5-HT receptors are coupled to second messengers; for example, 5-HT_{1A} receptors that modulate adenylate cyclase (Shenker *et al.* 1985; Markstein *et al.* 1986; DeVivo and Maayani, 1986) and 5-HT_2 receptors that activate phosphatidylinositol turnover (Brown *et al.* 1984; Conn & Sanders-Bush, 1985; Kendall and Nahorski, 1985). The combination of multiple responses mediated by a variety of receptors with different regional distributions makes the serotonergic system highly complex.

We have been studying the properties of specific synapses formed between identified leech neurones. The mechanosensory (P) cell receives serotonergic innervation *in situ* and in culture (Fuchs *et al.* 1982). Application of 5-HT by pipette elicits two long-lasting responses in the P cell: activation of an inhibitory Cl^- conductance and of an excitatory cation conductance (Henderson, 1983; Drapeau and Sanchez-Armass, 1988). Interestingly, only the Cl^- response is activated upon 5-HT release by the serotonergic Retzius cell (Fuchs *et al.* 1982; Drapeau and Sanchez-Armass, 1988), apparently because of the selective loss of the cationic response at sites of contact between the neurones (Drapeau *et al.* 1989).

In this study, we tested the effects of a variety of pharmacological agents in order to characterize the receptors that activate these ionic channels. This was particularly interesting since 5-HT receptors in invertebrate neurones (e.g. Walker

and Smith, 1973; Gerschenfeld and Paupardin-Tritsch, 1974; Drummond *et al.* 1980) have yet to be classified with the drugs developed recently for mammalian preparations.

The currents activated in the P cell by 5-HT released synaptically or by application by pipette are of long duration, lasting hundreds of milliseconds (Drapeau and Sanchez-Armass, 1988), which is common for 5-HT responses mediated by intracellular second messengers (Kehoe and Marty, 1980; Kaczmarek and Levitan, 1987). Therefore, an additional interest was to determine the nature of the long-lasting responses to 5-HT in the P cell. We show that the Cl^- and cationic responses occur in parallel through the actions of 5-HT on distinct receptors coupled to separate ionic channels *via* different second messenger pathways. A preliminary report of some of these results has appeared elsewhere (Drapeau & Sanchez-Armass, 1989).

Materials and methods

Cultures

P cells were isolated from the nervous system of the leech *Hirudo medicinalis* (purchased from Ricarimpex, Audenge, France) and cultured as described previously (Dietzel *et al.* 1986). Desheathed ganglia were exposed to collagenase (Type XI, Sigma Chemical Co., St Louis MO, USA) and the somata of P cells were removed by aspiration into a micropipette. The cells were plated (singly) in the wells of polylysine-coated microtest culture dishes containing Leibovitz-15 medium (L-15) supplemented with 2% heat-inactivated foetal bovine serum (Gibco Canada, Burlington, Ontario, Canada). Under these conditions, the cells usually did not extend neurites (which makes voltage-clamping easier; see Dietzel *et al.* 1986; Drapeau and Sanchez-Armass, 1988). Most experiments were performed 5–10 days following plating.

Recordings

P cells in culture were voltage-clamped with a single microelectrode (4 mol l^{-1} caesium acetate; 15–20 MΩ) using a sample-and-hold amplifier (Axoclamp 2a, Axon Instruments, Burlingame, CA) and were superfused continuously, as described previously (Drapeau and Sanchez-Armass, 1988). For experiments in which the pH of the superfusion solution was changed, a bath electrode was used to compensate for large voltage offsets. The solutions used were (concentrations in mmol l^{-1}): *Normal solution*, NaCl, 155; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 10; Hepes, 10, pH 7.4; *Calcium solution*, $CaCl_2$, 110; glucose, 10; Hepes, 10, pH 7.4; *TrisCl solution*, TrisCl, 165, pH 7.4; CsCl, 5; $MgCl_2$, 2; 3,4-diaminopyridine (DAP), 10; glucose, 10; *TEA^+ solution*, tetraethylammonium chloride (TEACl, prepared from 20% TEAOH titrated with HCl), 155; CsCl, 5; $MgCl_2$, 2; DAP, 10; glucose, 10; Mes, 10, pH 5.5 or 6.5, Hepes, 10, pH 7.4 or Tris, 10, pH 9.0 (for experiments at pH 5.5, Mes was used instead of Hepes); *Na_2SO_4 solution*, Na_2SO_4, 135; $MgSO_4$, 5; DAP, 10; glucose, 10; Hepes, 10, pH 7.4; anthracene-9-

carboxylic acid (9-AC: a Cl^- channel blocker), 0.1 mmol l^{-1} from a 0.4 mol l^{-1} stock solution dissolved in dimethylsulphoxide (DMSO). Other solutions containing a drug (e.g. antagonists or blockers) are indicated in the text and figure legends. Stock solutions of more hydrophobic drugs were prepared in DMSO and diluted so that the final DMSO concentration was no more than 0.05%, a concentration that had no direct effect on the recordings. 5-HT and other compounds (e.g. agonists or permeant second messengers) were applied by pressure ejection (200 or 500 ms pulses at 10^5 Pa) from a large-tipped (15–20 μm) pipette containing 100 μmol l^{-1} 5-HT or the indicated concentration of other compounds dissolved in the same solution used to superfuse the P cells. Switching solutions of the same ionic composition (but containing different drugs) had little effect on the holding current.

The compounds used and their sources are the following: 8-OH-DPAT [8-hydroxy-2(di-*N*-propylamino)tetralin], RBI; RU24969, gift of Roussel-Uclaf; spiroxatrine, Jansenn Pharmaceutica; metitepine, gift of Hoffmann-La Roche Ltd; ketanserin, gift of Dr C. deMontigny; ICS 205-930, gift of Sandoz Canada Inc.; cyproheptadine, forskolin, tolbutamide, dbcAMP (dibutyryladenosine-3′:5′-cyclic monophosphate) and dbcGMP (dibutyrylguanosine-3′:5′-cyclic monophosphate), Sigma Chemical Co.; amiloride, gift of J. Hanrahan; 3,4-dichlorobenzamil, gift of G. J. Kaczorowski; DPIB (12-deoxyphorbol-13-isobutyrate), PDA (phorbol-12,13-diacetate) and PMA (phorbol-12-myristate-13-acetate), LC Services Corp.; 1-(5-isoquinoline-sulphonyl)-2-methylpiperazine dihydrochloride (H-7) and *N*-[2-(methylamino) ethyl]-5-isoquinolinesulphonamide dihydrochloride (H-8), Seikagaku America, Inc., St Petersburg, FL.

Results

We describe below the chloride and cation conductances in the P cell activated by 5-HT. For each response the properties of the channels, their pharmacological profiles and the roles of different second messengers have been characterized.

The chloride conductance

Channel properties

We have described previously the Cl^- conductance (Drapeau & Sanchez-Armass, 1988). This response can be isolated by superfusing the P cell in TrisCl solution, an impermeant cation solution in which Na^+ and K^+ are replaced by Tris and Ca^{2+} by Mg^{2+}. The current–voltage relationship of the Cl^- conductance shows outward rectification and depends on the Cl^- gradient. The current is reduced by inhibitors of Cl^- channels, including 9-AC (Bryant and Morales-Aguilera, 1971) and the stilbene derivative 4,4-diisothiocyanostilbene 2,2-disulphonic acid (DIDS) (Knauf and Rothstein, 1971).

100 μmol l^{-1} Zn^{2+} reversibly reduced the Cl^- current (45±13% of control, N=7; Fig. 1A), as reported for other Cl^- channels (Bertag, 1987). At 10 μmol l^{-1}, Zn^{2+} had no significant effect (not shown). Since a variety of Cl^- channels are

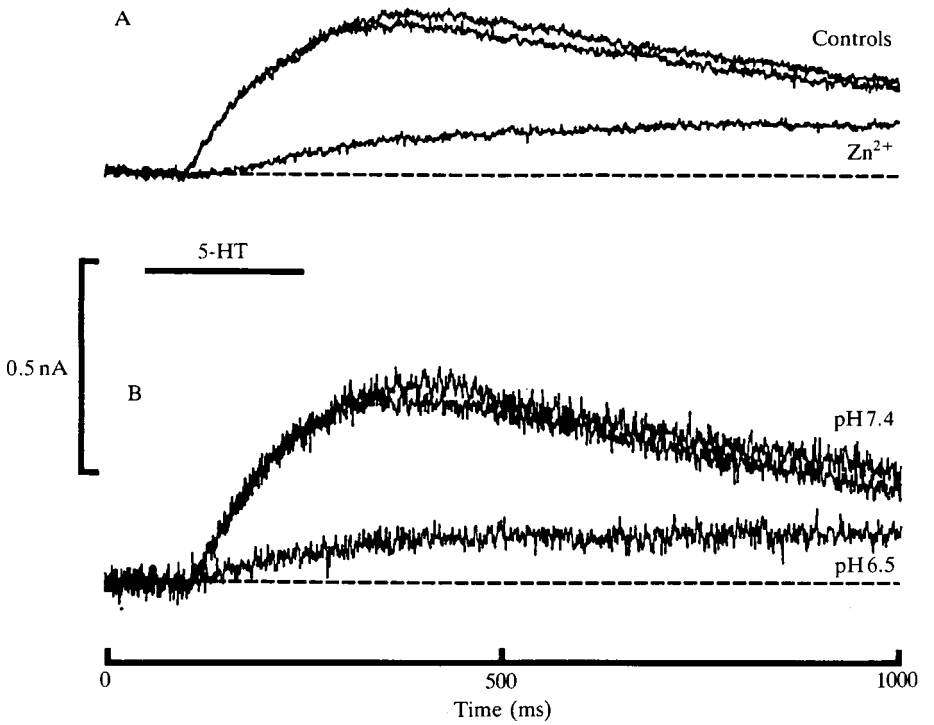

Fig. 1. Block of the Cl^- conductance by Zn^{2+} and low pH. (A) A cultured P cell was superfused with TrisCl solution with (Zn^{2+}) or without (Controls) 0.1 mmol l^{-1} $ZnCl_2$ present. (B) A different P cell was superfused with TEACl solution at pH 7.4 or 6.5, as indicated. For the period indicated by the bar, 100 μmol l^{-1} 5-HT dissolved in the same solution used to superfuse the P cell was pressure-ejected. The holding potential was 0 mV. The controls show the responses both before and after the treatments.

sensitive to the extracellular pH (Bretag, 1987), we examined the effects of acidification and alkalization on the Cl^- current. As shown in Fig. 1B, lowering the pH to 6.5 reversibly reduced the Cl^- current (35±15 % of control, N=7). The Cl^- current was completely blocked at pH 5.5 and was unaffected at pH 9.0 (not shown). Neither the block by Zn^{2+} nor that by H^+ was noticeably voltage dependent.

As described below, we tested the effects of a variety of compounds on currents elicited at different holding potentials (usually only one is shown) and the results were consistent with effects on the conductance to Cl^-

Pharmacology

5-HT agonists and antagonists were applied in order to characterise the receptor properties of the Cl^- conductance according to the criteria used for mammalian 5-HT receptor subtypes (Peroutka, 1988). Agonists were tested by pressure-ejecting TrisCl solution containing the drug while superfusing the P cell in drug-free TrisCl solution. Antagonists were tested on P cells superfused with TrisCl

solution containing the drug by pressure-ejecting TrisCl solution containing 100 μmol l^{-1} 5-HT (sufficient to give a maximal response in the absence of drug; Drapeau and Sanchez-Armass, 1988).

At low concentrations, 8-OH-DPAT and RU24969 are selective agonists of 5-HT_{1A} and 5-HT_{1B} receptors, respectively; at high concentrations, these compounds also activate other 5-HT_1 receptors, including 5-HT_{1C} and 5-HT_{1D} receptors (see Peroutka, 1988). Low concentrations of these drugs were ineffective and even very high concentrations (100 μmol l^{-1} 8-OH-DPAT or 200 μmol l^{-1} RU24969) failed to activate a significant Cl^- conductance (Fig. 2). When tested as antagonists, they failed to block the Cl^- conductance (not shown). High concentrations of spiroxatrine, a selective antagonist of 5-HT_{1A} receptors (Nelson and Taylor, 1986), and metitepine, which has the highest affinity of all antagonists

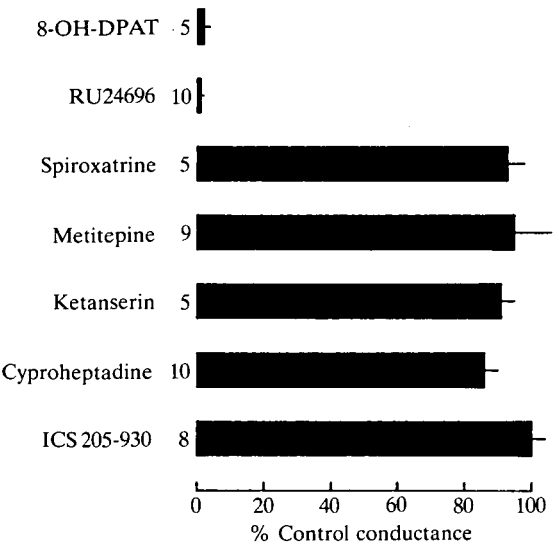

Fig. 2. Pharmacology of the Cl^- conductance. The effects of a variety of 5-HT-receptor-specific agonists and antagonists were tested on P cells superfused in TrisCl solution (P cells clamped at −20 to 0 mV). The effects of agonists (8-OH-DPAT and RU24969) were tested by pressure-ejecting a solution of each compound dissolved in TrisCl solution and comparing the responses to those elicited by ejection of a control solution containing 100 μmol l^{-1} 5-HT (mean of the control measurements obtained before and after testing each of the drugs). The effects of antagonists (spiroxatrine, metitepine, ketanserin, cyproheptadine and ICS 205-930) were tested by superfusing the cells with TrisCl solution containing the compound and measuring the relative amplitude of the current activated by pressure-ejection of 5-HT. The concentrations of the drugs were (μmol l^{-1}): 8-OH-DPAT, 100; RU24969, 200; spiroxatrine, 6; metitepine, 10; ketanserin, 10; cyproheptadine; 10; ICS 205-930, 10. The mean of all measurements of the peak conductances in the presence of a drug as a percentage of the mean of the control conductances elicited by 5-HT in the absence of (before and after) the test drug are illustrated by the bars in the histogram. The number of determinations and the S.E.M. are indicated for each bar.

for 5-HT_1 receptors (Leysen *et al.* 1981), failed to block the Cl^- conductance (Fig. 2). Therefore, the Cl^- conductance is not coupled to a 5-HT_1 receptor.

Micromolar concentrations of the highly selective 5-HT_2 antagonists ketanserin (Leysen *et al.* 1982) and cyproheptadine (McCall and Aghajanian, 1980) were also without effect on the Cl^- conductance. Higher, and therefore less specific, concentrations of cyproheptadine (100 μmol l^{-1} or more) can block the Cl^- conductance (Drapeau and Sanchez-Armass, 1988) and Cl^--dependent responses *in vivo* in the leech (Sawada and Coggeshall, 1976). These results argue against a 5-HT_2 receptor for the Cl^- conductance.

Recently, drugs specific for 5-HT_3 receptors have been developed (Peroutka, 1988). One of the first and best studied of these is ICS 205-930 (Richardson *et al.* 1985). As can be seen in Fig. 2, 10 μmol l^{-1} ICS 205-930 in the superfusion solution was without effect. In addition, 100 μmol l^{-1} ICS 205-930 applied by pipette did not elicit a response (not shown). These results argue against a role for a 5-HT_3 receptor in the Cl^- conductance.

In conclusion, the receptor mediating the effect of 5-HT on the Cl^- conductance does not have a pharmacological profile consistent with that of any of the mammalian classes of 5-HT receptors.

Second messengers

The activation of the Cl^- conductance by applied and synaptically released 5-HT is slow and long-lasting (Fuchs *et al.* 1982; Henderson, 1983; Drapeau and Sanchez-Armass, 1988). For example, the onset of the postsynaptic current in P cells innervated by serotonergic Retzius cells is delayed by about 10 ms following the presynaptic action potential, reaches a peak after 20–50 ms and declines along a biexponential time course with time constants of about 70 and about 600 ms (Drapeau and Sanchez-Armass, 1988). Sustained responses to 5-HT in neurones are generally due to the actions of second messengers (Kehoe and Marty, 1980; Kaczmarek and Levitan, 1987; Bobker and Williams, 1990). The effects on the Cl^- conductance of agents known to modify the activity of protein kinases were tested to determine the possible regulation by second messengers. An important criterion for the effects of activating compounds was that they should develop over a similar time course to the effects of 5-HT, i.e. with a delay of less than 100 ms and a prolonged effect following brief application.

Application of 1 mmol l^{-1} dbcAMP for 500 ms onto a P cell superfused in TrisCl solution resulted in the rapid activation of a prolonged current with a reversal potential near the resting potential (approx. −50 mV), similar to that observed for the Cl^- conductance (Drapeau and Sanchez-Armass, 1988). This effect was more pronounced when the phosphodiesterase inhibitor theophylline (1 mmol l^{-1}) was included with the dbcAMP (Fig. 3A). In addition to the Cl^- conductance, dbcAMP, but not 5-HT, activated a non-selective cation conductance permeant to Tris, unlike the cationic conductance described below, and impermeant to TEA^+ (S. Sanchez-Armass, in preparation). Consequently, the Cl^- conductance activated by dbcAMP was more apparent in TEA^+ solution (14/17 cells tested) than

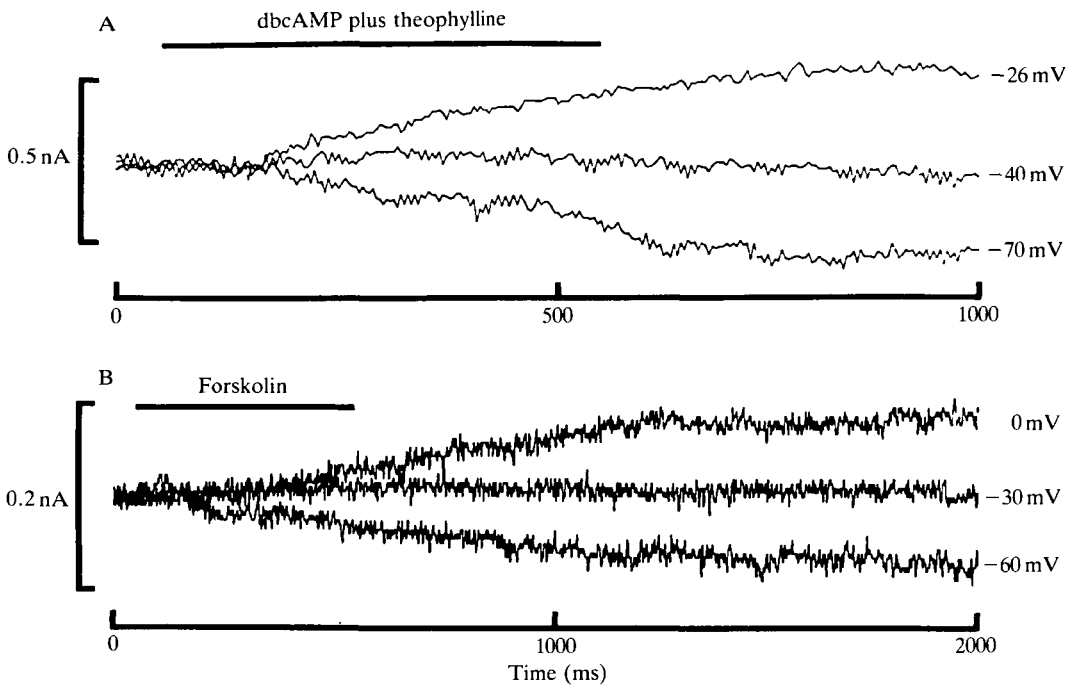

Fig. 3. Activation of the Cl^- conductance by dbcAMP and forskolin. P cells were superfused with TrisCl solution and voltage-clamped at the indicated potentials (current recordings were overlapped at the beginning of the traces). A solution of 1 mmol l^{-1} dbcAMP and 1 mmol l^{-1} theophylline (A) or 100 μmol l^{-1} forskolin (B) dissolved in TrisCl solution was pressure-ejected for the period indicated by the black bars.

in TrisCl solution (10/27 cells). Forskolin, which activates adenylate cyclase, also activated the Cl^- conductance in 15/23 cells in TrisCl solution (Fig. 3B) and 2/3 cells in TEA^+ solution.

To determine if the same Cl^- channels were being activated by 5-HT, dbcAMP and forskolin, we tested two blockers of cyclic-AMP-dependent protein kinase A. The first was tolbutamide, a sulphonylurea which blocks ATP-dependent K^+ channels (Trube *et al.* 1986) and protein kinase A (Kanamori *et al.* 1976) and which reversibly blocks cyclic-AMP-dependent processes including amylase secretion (Kanamori *et al.* 1974), hormone-stimulated lipolysis (Wray and Harris, 1973) and a Ca^{2+} current in snail neurones (Doroshenko *et al.* 1984). As shown in Fig. 4, tolbutamide reversibly reduced the Cl^- conductance activated by 5-HT (estimated $K_{0.5}$ of 0.5 mmol l^{-1}). In addition, the isoquinoline derivative H-8 (10 μmol l^{-1}), a non-selective inhibitor of protein kinases (Hidaka *et al.* 1984), reduced the Cl^- conductance activated by 5-HT (4±10 % of control, N=7), dbcAMP (20±19 % of control, N=5) and forskolin (29±14 % of control, N=8), as illustrated in Fig. 5. Therefore, the Cl^- conductance appears to depend on the activation of protein kinase A.

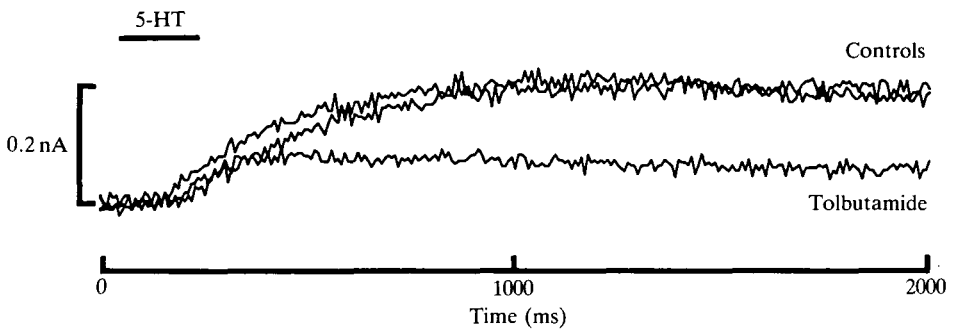

Fig. 4. Block of the Cl^- conductance by tolbutamide. P cells were superfused with TrisCl solution containing 1 mmol l^{-1} theophylline without (controls before and after superfusion of drug) or with 1 mmol l^{-1} tolbutamide and were voltage-clamped at 0 mV (current recordings were overlapped at the beginning of the traces). TrisCl solution containing 100 μmol l^{-1} 5-HT was pressure-ejected for the 200 ms period indicated by the black bar.

Application of 1 mmol l^{-1} dbcGMP failed to activate the Cl^- conductance (not shown). However, P cells superfused with TrisCl solution containing 100 μmol l^{-1} dbcGMP showed a weak inhibition of the response to 5-HT (72±8 % of control, N=15), suggesting that dbcGMP has a partial antagonistic effect on the activation of the Cl^- conductance by protein kinase A.

Application of up to 12 μmol l^{-1} of the phorbol esters DPIB or PDA, which activate protein kinase C (Nishizuka, 1988), failed to elicit the Cl^- conductance (not shown).

In conclusion, it appears that the Cl^- conductance is activated by the effect of 5-HT on an unindentified receptor subtype that is coupled to Cl^- channels *via* the activation of cyclic-AMP-dependent protein kinase A.

The cation conductance

Channel properties

In our previous study, the cation conductance was estimated to be relatively non-selective for monovalent (Na^+ and K^+) cations and did not require the presence of extracellular Ca^{2+} (Drapeau & Sanchez-Armass, 1988). As shown in Fig. 6, 5-HT did not elicit an inward current in isotonic $CaCl_2$ (for all 12 P cells tested), demonstrating that the cation conductance was impermeant to this divalent cation. We tested the effects of amiloride and a related compound, 3,4-dichlorobenzamil, as blockers of the cation conductance, since micromolar concentrations of amiloride block certain Na^+ channels in a number of preparations (Garty and Benos, 1988). As shown in Fig. 7, micromolar concentrations of amiloride reduced the cation conductance. 3,4-Dichlorobenzamil had the same effect (not shown).

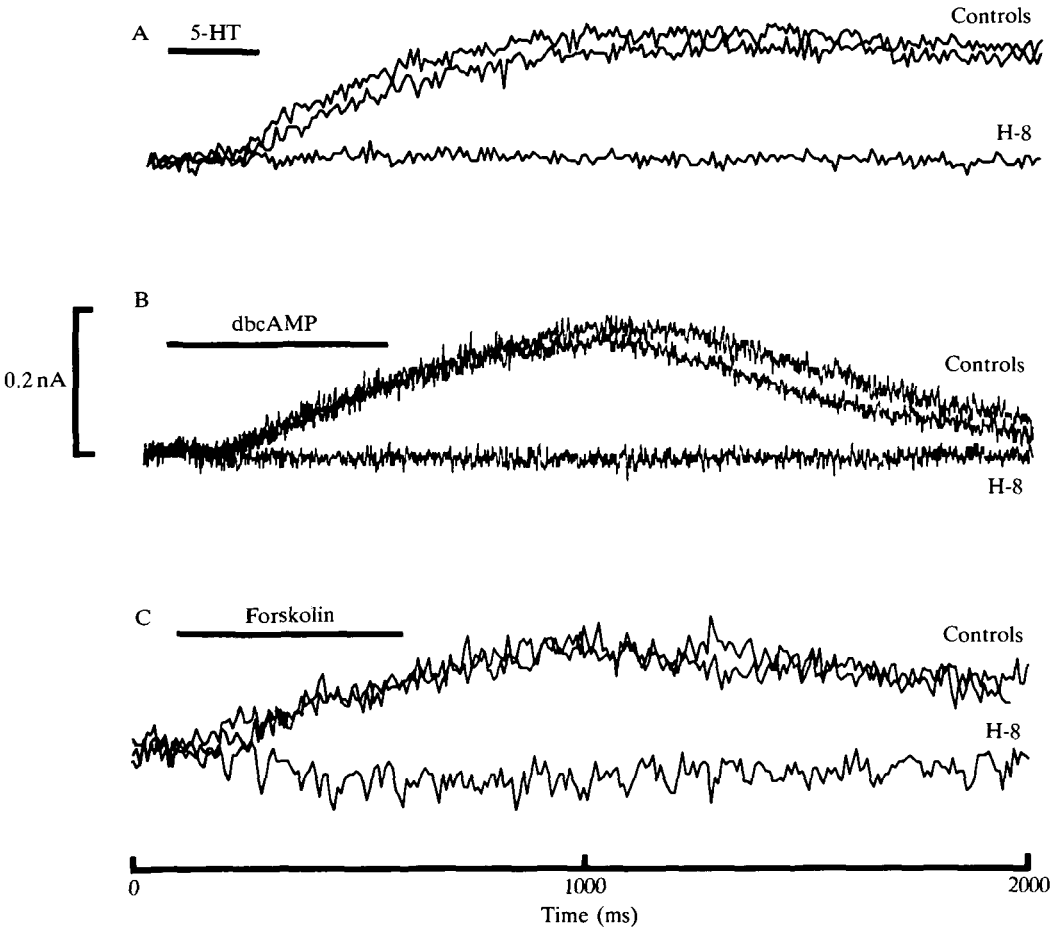

Fig. 5. Block of the Cl^- conductance by H-8. The experimental protocol was identical to that described in the legend to Fig. 4 except that 10 μmol l^{-1} H–8 was used (instead of tolbutamide) in one of the solutions. TrisCl solution containing 100 μmol l^{-1} 5-HT (A), 1 mmol l^{-1} dbcAMP (B) or 100 μmol l^{-1} forskolin (C) was pressure-ejected for the 200 ms period indicated by the black bar.

Pharmacology

As observed for the Cl^- conductance, the 5-HT_1 receptor agonists 8-OH-DPAT and RU24969 and antagonists spiroxatrine and metitepine and the 5-HT_3 receptor antagonist ICS 205-930 were ineffective when tested on the cation conductance (Fig. 8B). However, micromolar concentrations of the 5-HT_2 antagonists ketanserin (Fig. 8A,B) and, to a lesser extent, cyproheptadine (Fig. 8B) reduced the cation conductance. Higher concentrations of cyproheptadine (100 μmol l^{-1} or more) resulted in complete block of the cation conductance (Drapeau and Sanchez-Armass, 1988). Therefore, the cation conductance is coupled to a receptor resembling pharmacologically the mammalian 5-HT_2 subtype.

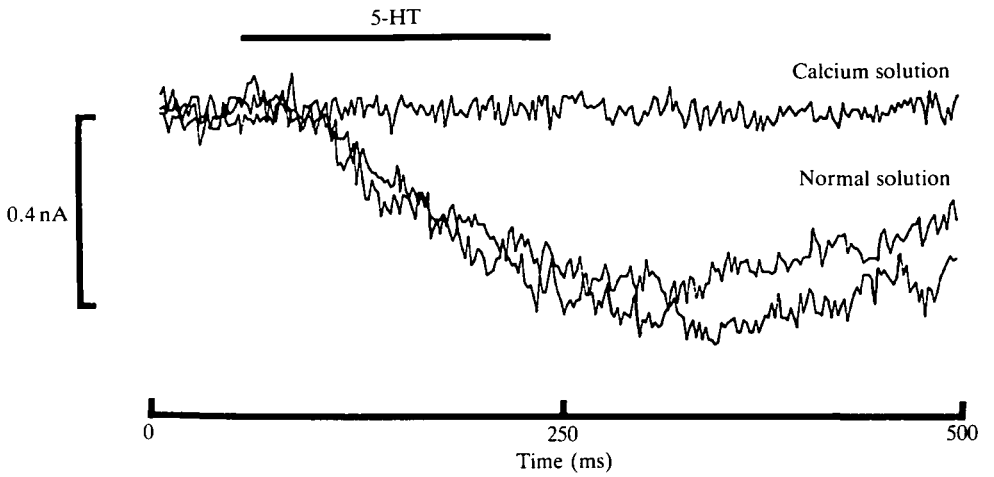

Fig. 6. Permeability of the cation conductance for Na^+ and Ca^{2+}. The P cell was superfused with either normal solution or calcium solution and held at −60 mV. 5-HT dissolved in the same solution used for superfusion was ejected for the 200 ms period indicated by the black bar.

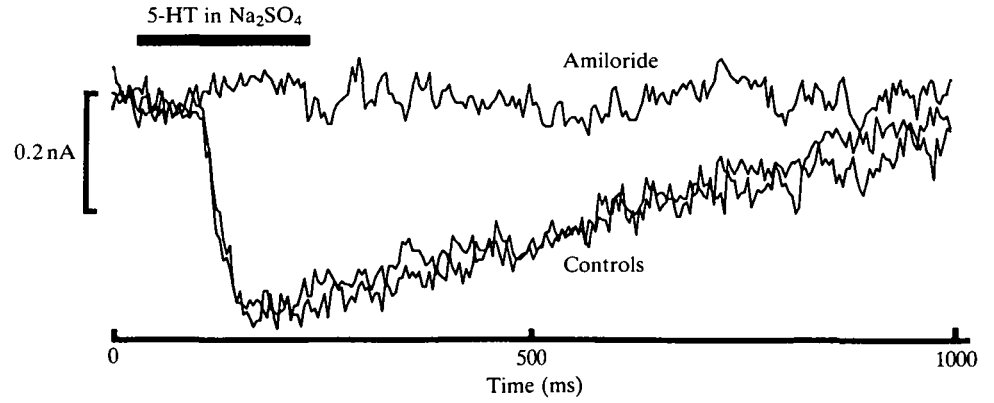

Fig. 7. Block of the cation conductance by amiloride. A P cell was superfused with Na_2SO_4 solution and voltage-clamped at −60 mV in the absence (controls before and after drug treatment) and in the presence of 3 μmol l^{-1} amiloride. For the 200 ms period indicated by the black bar, 100 μmol l^{-1} 5-HT dissolved in Na_2SO_4 solution was pressure-ejected onto the P cell. The current traces were overlapped at the beginning of the recordings.

Second messengers

We tested the effects of a brief application of modifiers of protein kinase activities on the cation conductance. Pressure ejection of 1 mmol l^{-1} solutions of the dibutyryl derivatives of either cyclic AMP or cyclic GMP did not appear to activate the cation conductance (not shown). In addition, superfusing the P cells

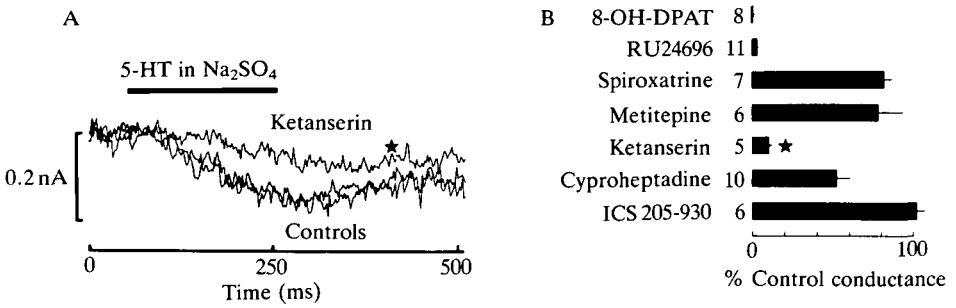

Fig. 8. Pharmacology of the cation conductance. (A) A P cell was superfused with Na_2SO_4 solution and voltage-clamped at −60 mV in the absence (controls before and after drug treatment) and in the presence of 1 μmol l^{-1} ketanserin. For the 200 ms period indicated by the black bar, 100 μmol l^{-1} 5-HT dissolved in Na_2SO_4 solution was pressure-ejected onto the P cell. The current traces were overlapped at the beginning of the recordings. (B) Agonists and antagonists were dissolved in Na_2SO_4 solution and tested as described for Fig. 2. The number of measurements and S.E.M. are indicated for each group. The P cells were clamped at −40 to −70 mV. The stars indicate the results obtained for ketanserin reduction of the cation current in a single experiment (A) and for the mean of several experiments (B).

with Na_2SO_4 solution containing 0.6 mmol l^{-1} tolbutamide, which was sufficient to reduce the Cl^- conductance to about one-third of the control value (Fig. 4), did not block the cation conductance significantly (87±4 % of control, N=7).

Brief (<1 s) application of micromolar concentrations of the less hydrophobic phorbol esters DPIB (1 μmol l^{-1}; Fig. 9) and PDA (12 μmol l^{-1}; not shown), which directly activate protein kinase C (Nishizuka, 1988), elicited an inward current at hyperpolarized potentials in P cells superfused with Na_2SO_4 solution. We examined whether the cationic current activated by the phorbol esters underlies the cation conductance activated by 5-HT by testing the ability of amiloride to block the DPIB-activated current. As shown in Fig. 9, a high (20 μmol l^{-1}) concentration of amiloride partially reduced the effects of DPIB (57±15 % of control, N=4), suggesting that the phorbol esters activated the same cation conductance as well as an amiloride-insensitive component. The compound H-7 (50 μmol l^{-1}), a high-affinity blocker of protein kinase C (Kawamoto and Hidaka, 1984), almost completely reduced the cation conductance activated by 5-HT (10±12 % of control, N=13) or phorbol esters (21±17 %, N=3), as illustrated in Fig. 10. The less specific inhibitor H-8, which was a potent blocker of the Cl^- conductance (Fig. 5), failed to block the cation conductance at concentrations up to 10 μmol l^{-1} (not shown). Higher concentrations (10 μmol l^{-1}) of phorbol esters activated a much larger current that could result in loss of clamp control and the appearance of active currents.

Recently, single cation channels recorded in P-cell-attached patches were shown to be activated by both 5-HT and phorbol ester, and H-7 was shown to block these effects (Drapeau, 1990). The results with macroscopic and single-channel record-

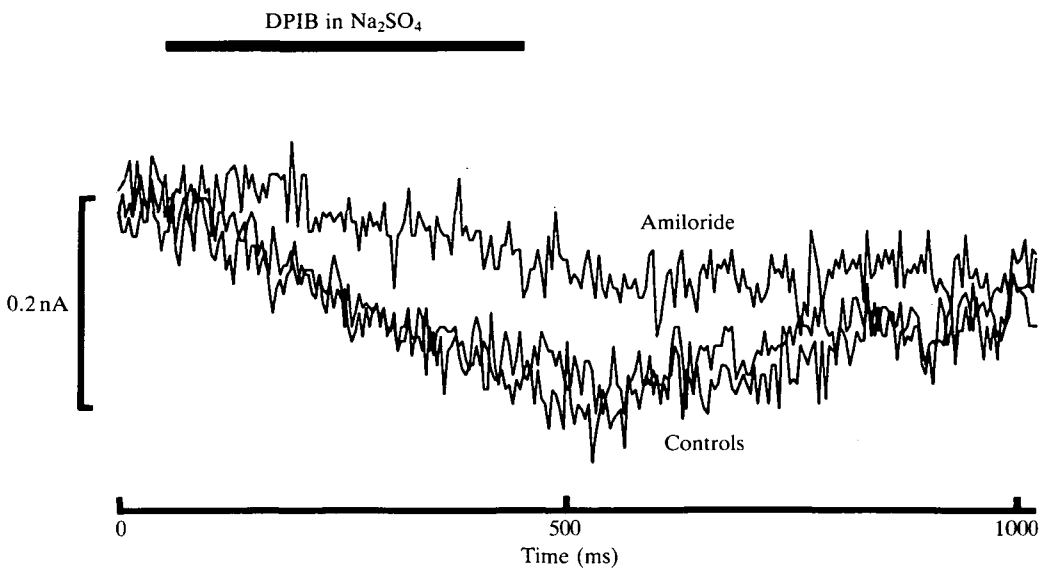

Fig. 9. Activation of the cation conductance by phorbol ester. A P cell was superfused with Na_2SO_4 solution and voltage-clamped at −45 mV in the absence (controls before and after treatment) and in the presence of 20 μmol l^{-1} amiloride. For the 400 ms period indicated by the black bar, 1 μmol l^{-1} DPIB dissolved in Na_2SO_4 solution was pressure-ejected onto the P cell. The current traces were overlapped at the beginning of the recordings.

ings together suggest that the cation conductance is linked to a 5-HT_2 receptor and depends on activation of protein kinase C.

Discussion

Our results demonstrate that 5-HT activates two distinct receptors coupled to separate ionic channels *via* different second messenger pathways. The Cl^- channels were blocked by Zn^{2+} and H^+, consistent with the results obtained, in a wide variety of species and tissues, for Cl^- channels which are open in the absence of a specific ligand (Bretag, 1987). In contrast, the GABA receptor/Cl^- channel complex in lobster muscle is blocked by Zn^{2+} and is activated by H^+ (Smart and Constanti, 1982). As discussed below, the P cell Cl^- channels are operated by a second-messenger system, supporting the possibility that the Cl^- channels are physically distinct from the 5-HT receptors.

The Cl^- conductance was insensitive to a variety of drugs that affect 5-HT receptors in mammalian preparations, suggesting that the receptor may be of a novel type, perhaps restricted to invertebrates. Clear identification of the receptor activating the Cl^- conductance as a different receptor type would require the development of specific agonists or antagonists. As observed for some 5-HT responses in other preparations (Kehoe and Marty, 1980; Kaczmarek and Levitan,

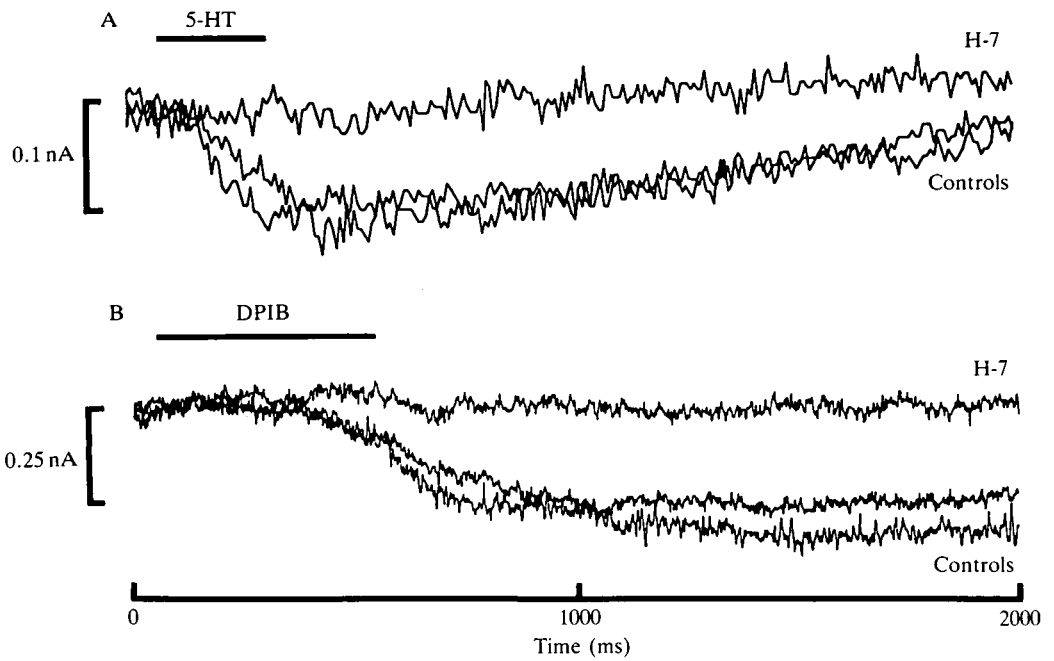

Fig. 10. Block of the cation conductance by H-7. P cells were voltage-clamped and superfused with Na_2SO_4 solution without or with 50 μmol l^{-1} H-7. 5-HT (100 μmol l^{-1}, holding potential of −46 mV) or DPIB (0.1 μmol l^{-1}, holding potential of −42 mV) dissolved in the superfusion solution was pressure-ejected onto the cell for the indicated period. Controls show the responses before and after treatment.

1987; Bobker and Williams, 1990), the Cl^- conductance was apparently dependent on the action of cyclic AMP, presumably requiring activation of protein kinase A. An association between adenylate cyclase and 5-HT_{1A} receptors has been suggested from studies of the mammalian brain (Shenker *et al.* 1985; Markstein *et al.* 1986; De Vivo and Maayani, 1986). Our results suggest that the receptor activating the Cl^- conductance may be an invertebrate equivalent of the 5-HT_{1A} receptor that is insensitive to the drugs developed for mammalian preparations. It is interesting that a 5-HT response in *Aplysia* neurones that is dependent upon cyclic AMP is blocked by the 5-HT_2-specific antagonists ketanserin and ritanserin (Ocorr and Byrne, 1986), again at odds with mammalian pharmacology.

A cyclic-AMP-dependent pathway would explain the prolonged effects of the synaptic activation of the Cl^- conductance in the P cell (Drapeau and Sanchez-Armass, 1988). Cl^--dependent miniature synaptic potentials are observed at the Retzius–P cell synapse (Henderson *et al.* 1983; Dietzel *et al.* 1986). As for the secretion of transmitters having rapid, direct effects on postsynaptic channels (e.g. the acetylcholine receptor/channel), our results suggest that second-messenger-dependent responses can occur by quantal release of transmitter.

The cation response fits more readily into the mammalian scheme as a 5-HT_2

receptor. A difference between the cation conductance and mammalian 5-HT_2 receptors was the lack of effect of metitepine on the cation conductance, since this antagonist binds with high affinity to both 5-HT_1 and 5-HT_2 receptors (Leysen *et al.* 1981). The activation of the cation conductance by phorbol esters indicates a role for diacylglycerol, a product of inositide phospholipid metabolism which activates protein kinase C (Nishizuka, 1988) and ionic channels (Berridge, 1986). The blockage of the cation conductance by H-7 is also consistent with a role for protein kinase C in activation of the channel. This provides a further similarity between the cation conductance and mammalian 5-HT_2 receptors, since this receptor type in rat brain is believed to be coupled to phosphoinositide turnover (Brown *et al.* 1984; Conn and Sanders-Bush, 1985; Kendall & Nahorski, 1985).

We have shown that, during innervation of the P cell by the serotonergic Retzius cell in culture, contact between the neurones causes the selection of the correct *in vivo* response, i.e. only the Cl^- conductance and not the cation conductance is activated upon 5-HT secretion (Drapeau *et al.* 1989). Recordings of single cation channels suggest that it is the activation of the channels by protein kinase C that is reduced as a prelude to synapse formation (Drapeau, 1990). The existence of parallel mechanisms for processing these responses in the P cell appears to be critical to the selection process, since the independent pathways permit the inactivation of one response without affecting the other.

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