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5-HT 2 and 3 Agonists Influence the Contraction Activity of the Auricles from the Central Heart Complex of *Sepia officinalis* L. (Cephalopoda)

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Tobias Lehr and Rudolf Schipp (2005) 5-HT 2 and 3 agonists influence the contraction activity of the auricles from the central heart complex of *Sepia officinalis* L. (Cephalopoda). *Zoological Studies* **44**(4): 468-474. In this study we investigated the effects of specific 5-HT_2 and 5-HT_3 agonists on the contraction activity of isolated auricles from the cuttlefish *Sepia officinalis*. We show that the specific 5-HT_2 agonists, m-CPP and α-CH_3-5-HT, increased the contraction force of the auricles and that the effect of α-CH_3-5-HT was blocked by the selective 5-HT_2 antagonist ketanserin, the membrane-permeable phospholipase C inhibitor U-73122 and the membrane-permeable D-myo-inositol 1,4,5-trisphosphate (IP_3) receptor antagonist 2-aminoethoxy-diphenylborane (2-APB). These results suggest that a subtype of the 5-HT_2-like receptor seems to be involved in regulation of the contraction force of the auricle. The blocking effect of the membrane-permeable enzyme inhibitors of the 5-HT_2-specific signal transduction enzymes confirms that the cell response is triggered by the phosphatidylinositol-response including phospholipase C activation and the IP_3-dependent signal transduction pathway in the auricular myocardium. In addition, we show that the specific 5-HT_3-agonists 1-phenylbiguanide (1-PBG), m-chlorophenylbiguanide (m-CPBG) and 2-CH_3-5-HT evoked concentration-dependent excitatory effects on the isolated auricles. While all tested 5-HT_3 agonists increased the frequency of the auricles, only 2-CH_3-5HT increased the contraction force of the auricles, and only 1-PBG caused positive effects on the tone of the isolated auricles. These results suggest that except for a 5-HT_2-like receptor, signal transduction mechanisms sensitive to 5-HT_3 agonists, and possibly an ion channel receptor are involved in the neuroregulation, primarily modulation of the heart frequency, of *S. officinalis* auricles. http://www.sinica.edu.tw/Journals/44.4/468.pdf

Key words: Mollusca, PI-response, Ion channel, PLC, *Sepia*.

Characterization of the 5-HT receptor and its subtypes within invertebrates has been the subject of numerous physiological studies. Molluscs have frequently been used as model species for invertebrates. For example, in tissues from *Aplysia californica*, the presence of a 5-HT_2 receptor subtype was recently reported (Barbas et al. 2002). Physiological and molecular biological investigations on *Lymnaea stagnalis* also suggested the existence of a 5-HT receptor with 5-HT_2 properties (Walcourt-Ambakederemo and Winlow 1994, Gerhardt et al. 1996). In earlier studies on the systemic heart of *Sepia officinalis*, it was found that 5-HT has an excitatory effect on the auricles and the ventricle; the effects on the auricles could be inhibited by the 5-$HT_{1,2}$ agonist mianserin, but not by the 5-HT_2 antagonist cyproheptadin (Versen 1999). More recent results showed that 5-HT mainly induces excitatory effects on the auricles of *S. officinalis* which can be blocked by the specific 5-HT_{1a} antagonist NAN-190 (Lehr and Schipp 2004a). Furthermore, these studies suggested that an antagonistic 5-HT receptor system with 5-HT_1- and 5-HT_4-like properties regulates the auricular contractions generating excitatory and inhibitory effects on frequency and tone (Lehr and

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Schipp 2004b). The present physiological study focuses on investigating whether 5-HT_2-like receptor binding sites are present in the auricles of *S. officinalis*. Earlier investigations of the *Sepia*-auricles showed that the phospholipase C (PLC) inhibitor U-73122 was able to restrain noradrenaline-caused excitatory auricular activity of *S. officinalis* (Versen 1999) providing the first indication that the PLC seems to be involved in the cell signal transduction pathway. The role of the G-protein-coupled mechanism and its enzymes is well described in molluscs (Rack et al. 1992, Li et al. 1995, Suzuki et al. 1999, Kawasaki et al. 2004) as well as in other invertebrates (Tierney 2001). In vertebrates, the 5-HT_2 receptor is a G-protein-coupled receptor that acts via the well-known phosphatidyl-inositol-response activating PLC, which liberates the second messengers DAG and IP_3 from the membrane phospholipid 1,4-bisphosphoglyerate (Berridge et al. 1983, Berridge and Irvine 1984, Berridge 1987). While there are numerous findings about 5-HT_1 and 5-HT_2-like receptors in invertebrates as mentioned, data on the presence of 5-HT_3-like receptors in invertebrates are still scare. This receptor subtype in vertebrates is known to be an ion channel which passes sodium, potassium and calcium ions (Fozard 1989, Jackson and Yakel 1995, Higgins and Kilpatrick 1999). Some investigations described the action of 5-HT on ion channel binding sites on neurons of *L. stagnalis* (Walcourt-Ambakederemo and Winlow 1995) and on neurons of the visceral ganglion of *Helix aspersa* (Green et al. 1996) suggesting the presence of a subtype of a 5-HT_3-like receptor in molluscan tissues. Data on a 5-HT_3-like receptor in cardiac organs of molluscs, especially cephalopods, do not exist. Thus, it was a further aim of the present study to investigate the action of specific 5-HT_3 agonists on the isotonically suspended auricles of *S. officinalis*.

MATERIAL AND METHODS

For pharmacological bioassays, 20 semi-adult *Sepia officinalis* L. (with mantle lengths of 60-120 mm) of both sexes were used. The animals were caught from the Bassin d'Arcachon (Atlantic Ocean), were kept in circulated, aerated tanks and were fed daily for 3 days before being used for the bioassays. Before surgery, they were anesthetized in a 3% (v/v) ethanolic seawater (SW) solution. Animals were considered anesthetized when the tentacles ceased to move, and no postural reflex occurred when the animal was turned on its back. The legal requirements for animal care were adhered to.

Bioassays of isolated auricle preparations

For the bioassays, isolated auricles (left or right ones, lengths: 2-3 mm) were stored in physiological solution (iso-osmotic filtered SW with 0.17% glucose (w/v); pH=8.2) at 6-8°C for 4-7 h before the start of the experiment, to certify that no intrinsic transmitters persisted. Next, the preparations were equilibrated to room temperature, mounted on stainless-steel clamps and isotonically suspended in a 50 ml water-jacketed organ bath (18 °C) with 1 clamp anchored and the other fixed to a vertical, freely moveable lever connected to a strain gauge. The pressure transducer was connected to a direct-current bridge amplifier and its signals were registered on a 2-channel inkjet-recorder. The standardized equipment was calibrated by hanging a standard mass on the lever (1 cN). Depending on the auricular size, they were suspended with 0.8 to 1.5 cN before the experiment was started. The initial resting tension was adjusted until regular and constant activity (15-20 min.) was achieved. Drugs were applied using the cumulative dose-dependent method as described by van Rossum (1963). After each drug application, a pause lasting up to 10 min. (or until a stable organ response had occurred) was followed by incubation. In experiments with an antagonist or enzyme inhibitor, these substances were first applied at a constant concentration of 10^{-6} M in order to check for a possible inherent effect, and second, to check for a possible displacement by an increasing agonist concentration of 10^{-5} M applied afterwards. Contractions of untreated organs in physiological solution were recorded as a reference (SW-value) for the calculation of the concentration/response curves (based on fitting the data to Hill's 4-parameter equation; Endrenyi 1981). Drug responses were expressed as the percentage deviation from the reference values (mean ± S.E.M.; n is the number of rated preparations). For calculating the frequency, peaks were counted for a time unit, whereas for calculating of the amplitude, the change in peak height was measured. For tone calculations, the change from the baseline of the untreated stable organ response was measured. Finally, the half-maximal excitatory concentration (EC_{50}) and the pD_2 (-log EC_{50}) values were calculated for each concentration/response curve, if possible, as previously

described (van Rossum 1963). Significant differences between values were estimated using Student's *t*-test for paired comparisons and significance was assumed at a $p \leq 0.05$. In the figures, values are presented as percentage deviation from the control values as described in detail by Lehr and Schipp (2004a,b). The following drugs were used: α-CH_3-5-HT (5-HT_2-agonist, Tocris), m-CPP (5-HT_2-agonist, Sigma), 1-phenylbiguanid (-PBG, 5-HT_3-agonist, Tocris), 2-CH_3-5-HT (5-HT_3-agonist, Tocris), m-chlorphenylbiguanid (m-CPBG, 5-HT_3-agonist, Tocris), ketanserin tartrate (5-HT_2-antagonist, Sigma), 2-APB (Ca^{2+}-sensitive IP_3-channel inhibitor, Tocris) and U-73122 (PLC-inhibitor, Tocris).

RESULTS

All auricles showed spontaneous contractions after surgery, storage time and reacclimation to room temperature. The contraction rate of the auricles was not influenced by isotonic stretching of the organ, and no antagonist or inhibitor had any effect on the contractile activity. After suspending the auricles with the standardized equipment, as described above, and allowing the auricles to equilibrate for period of 15-20 min., they showed stable actograms with a regular rhythm of 3 ± 1 beats min^{-1} (Fig. 1).

Effects of the 5-HT_2 agonists m-CPP and α-CH_3-5-HT

The 5-HT_2 agonist m-CPP tested on 3 auricles had only a weak inhibitory effect on the frequency of auricular contractions with a maximum of -15.12 ± 9.07 %, when the highest concentration of 10^{-5} M was applied. However, the contraction force was increased by m-CPP and rose up to a maximum of 98.05 ± 7.25 % (Fig. 2). The EC_{50} value of the contraction force, evaluated from the concentration/response curve, amounted to 1.38 x 10^{-8} M (pD_2 = 7.86). The effects on the tone caused by m-CPP were non-uniform, such that the tone in the concentration/response curve is not shown and no experiment in combination with an antagonist was performed. The 5-HT_2 agonist α-CH_3-5-HT, tested on 10 auricles, mainly influenced the contraction force up to a maximum of 95.38 ± 9.06% (pD_2 = 7.3). In the beginning, α-CH_3-5-HT concentrations up to 10^{-6} M induced a decrease in auricle tone which changed at the highest concentration of 10^{-5} M into a rapid increase in auricle tone up to a maximum of 127.5 ± 25.9% (pD_2 = 4.4) (Figs. 3, 4A). The frequency of the auricles was not influenced by the agonist as strongly as were the amplitude and the tone. The contraction force increased in a concentration-dependent

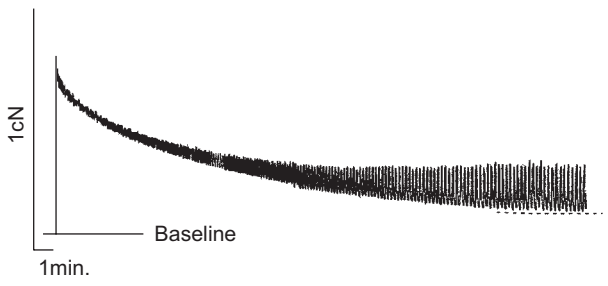

Fig. 1. Illustration of the auricular contraction activity after equilibration (baseline = above the pressure sensor registers a signal).

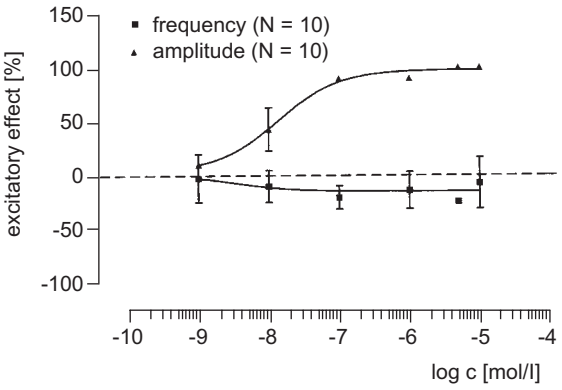

Fig. 2. Concentration/response curve of the 5-HT_2 agonist m-CPP on the isotonically suspended auricle. Values are shown as the mean ± S.E.M.

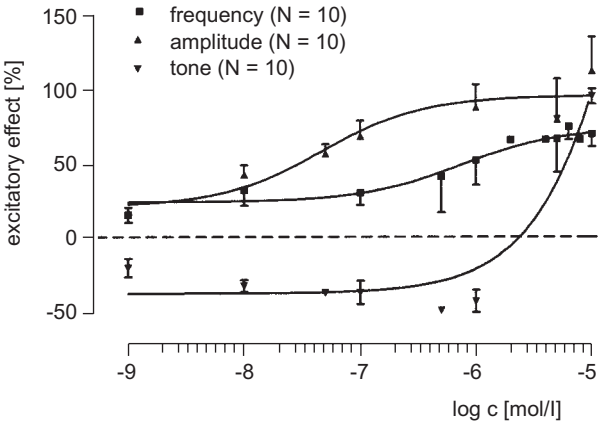

Fig. 3. Concentration/response curve of the 5-HT_2 agonist α-CH_3-5-HT on the isotonically suspended auricle. Values are shown as the mean ± S.E.M.

manner and reached its maximum at 74 ± 11.04% (Fig. 3).

Influence of ketanserin

In the presence of the 5-HT_2-specific antagonist ketanserin, the excitatory effect of α-CH_3-5-HT on increasing the contraction force was not very strong but was significantly reduced ($p < 0.05$), reaching a maximum of 59.2 ± 3.77% (Figs. 4B, 5). Ketanserin was also able to significantly ($p < 0.05$) block the strong positive effect on the frequency. The excitatory effect on tone, caused at a α-CH_3-5-HT concentration of 10^{-5} M, was not significantly blocked ($p > 0.05$).

Influence of the membrane-permeable enzyme inhibitors U-73122 and 2-APB

The IP_3-sensitive channel inhibitor 2-APB and the PLC-inhibitor U-73122 caused similar antagonistic effects. In the presence of U-73122 contractions were downregulated by increasing α-CH_3-5-HT concentrations to a minimum of -8.55 ± 19.7%, which means that there was a reversal effect (Fig. 4D). In the presence of the inhibitor of the IP_3-sensitive Ca^{2+} channel, 2-APB, the same effect was observed as in the presence of U-73122 but even more potently, meaning, that the contraction force was down-regulated to a value of -49.42 ± 9.21% in comparison with the reference value for SW (Fig. 4C). With both enzyme inhibitors, a total reversal of auricular contractions was measured (Fig. 5). The inhibitory action on the positive α-CH_3-5-HT effects on tone in the presence of 2-APB was similar, but not as strong, thus no concentration/response curve is shown. Neither 2-APB nor U-73122 had an inhibiting effect on the positive increase in the auricular beating frequency evoked by α-CH_3-5-HT.

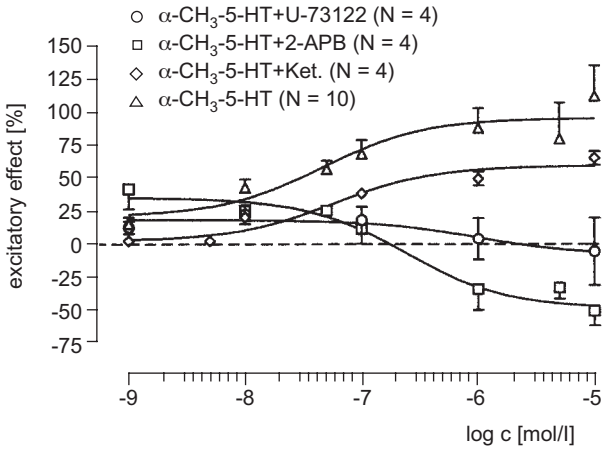

Fig. 5. Concentration/response curves for the influence of increasing α-CH_3-5-HT concentrations on the auricle in presence of the specific 5-HT_2 antagonist ketanserin (Ket.) the IP_3-sensitive Ca^{2+}channel inhibitor 2-APB and the PLC-inhibitor U-73122 (all at a constant concentration of 10^{-6} M). Values are shown as the mean ± S.E.M.

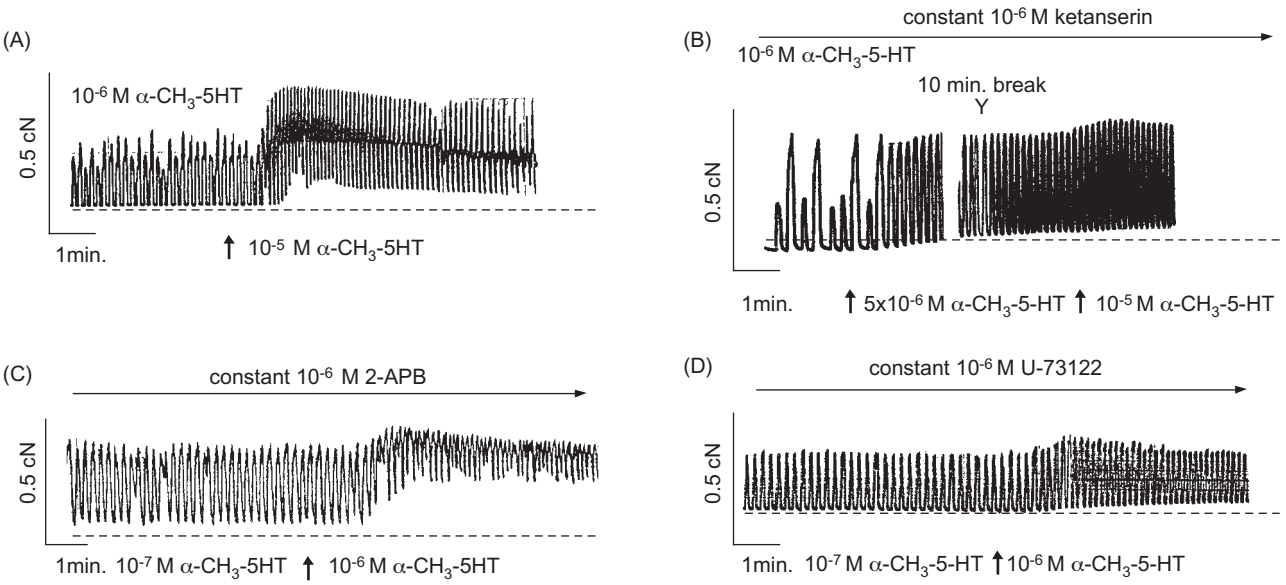

Fig. 4. Comparative illustrations of the effect of increasing α-CH_3-5-HT concentrations on the auricle alone (A) and in presence of the specific 5-HT_2 antagonist ketanserin (B), the IP_3-sensitive Ca^{2+} channel inhibitor 2-APB (C) and the PLC-inhibitor U-73122 (D) (all applied at a constant concentration of 10^{-6} M).

The action of 5-HT_3 agonists

The specific 5-HT_3 agonists 1-PBG, m-CPBG and 2-CH_3-5-HT were used to examine the response to putative 5-HT_3 binding sites within the auricular tissue. All 3 agonists evoked positive effects on auricle activity, but to different degrees (Figs. 6A-C). The maximal frequency values (EC_{100}) were reached at concentrations of 10^{-5} M for each agonist and amounted to: 50.24 ± 5.56% (pD_2 = 8.3) for 1-PBG, 25.68 ± 12.6% (pD_2 = 6.36) for 2-CH_3-5-HT and 72.33 ± 9.23% (pD_2 = 7.65) for m-CPBG. Under the influence of 2-CH_3-5-HT, the contraction force increased in a concentration-dependent manner to a maximum of 89 ± 25% (pD_2 = 5.76) (Fig. 6C). The other 2 agonists evoked only inhibitory effects on the contraction force (Fig. 6A, B). The effects of the 3 agonists on the tone differed. Only 1-PBG caused positive action on the tone of isolated auricles with a maximum of 31.75 ± 4.8% (pD_2 = 7.66) at a concentration of 10^{-5} M (EC_{100}) (Fig. 6A). The other 2 agonists showed negative effects on the tone at concentrations of 10^{-5} M (EC_{100}) with values of -13.16 ± 4.47% (pD_2 = 8.86) for m-CPBG and -18.27 ± 4.43% (pD_2 = 7.1) for 2-CH_3-5-HT.

DISCUSSION

The cloning of a 5-HT receptor subtype of *Lymnaea stagnalis*, which showed similarities of its pharmacological profile to that of a 5-HT_2 receptor in vertebrates, provided the first evidence that 5-HT_2-like receptors are expressed in a mollusc (Gerhardt et al. 1996). These findings corresponded to studies on the systemic heart of the molluscan species *S. officinalis* which demonstrated an increase in the contractile activity in auricles by 5-HT_2 agonists indicating the existence of a 5-HT_2-like receptor. Findings of the present study suggest that the excitatory action on the auricles of *S. officinalis* evoked by the 5-HT_2 agonists m-CPP and mainly α-CH_3-5-HT are caused by activation of the IP_3/DAG-dependent second messenger signal transduction pathway. Evidence for this can be seen in the restraining effect of the antagonists used such as ketanserin, a PLC-inhibitor (U-73122) and an IP_3-inhibitor (2-APB). All three of them significantly inhibited the excitatory effect of α-CH_3-5-HT on the contractile activity of the auricles. Investigations on the molluscan species *L. stagnalis* (neurons) and *Archachatina marginata* (retractor muscle) previously showed that ketanserin is a useful drug for blocking the effects of α-CH_3-5-HT and 5-HT (Innocent and Olufemi 1992, Walcourt-Ambakederemo and Winlow 1994). The strong increase in the contraction force in the auricle of *S. officinalis* induced by α-CH_3-5-HT could be explained by an increasing concentration of intracellular Ca^{2+}, released

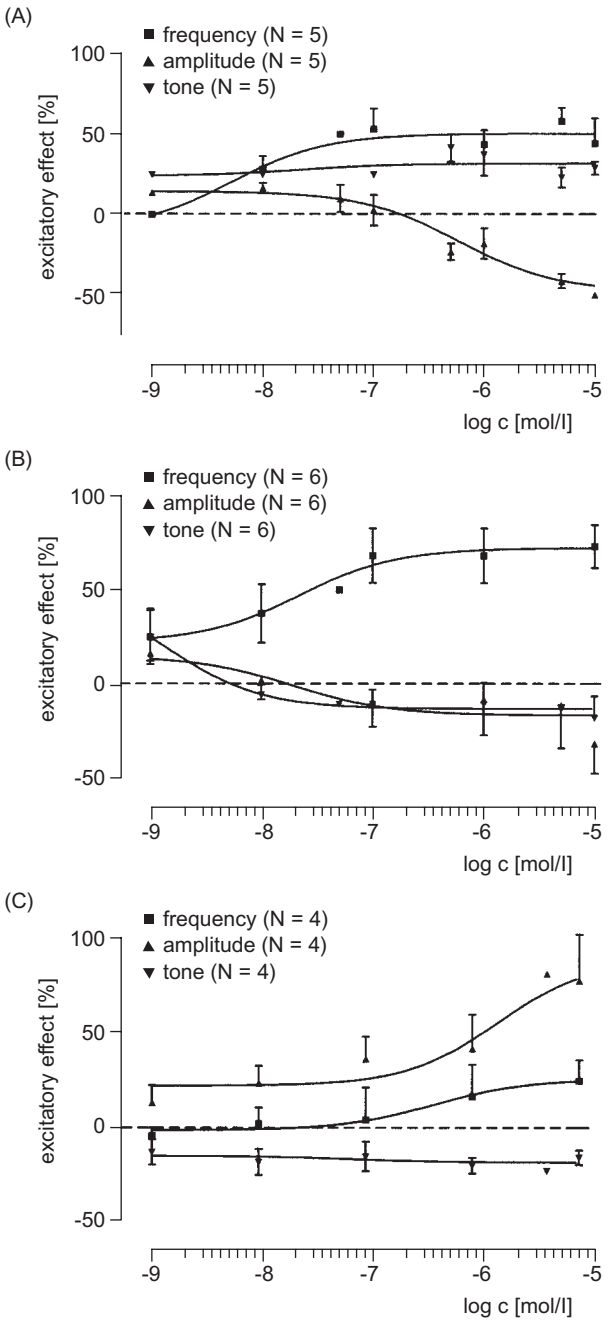

Fig. 6. Concentration/response curves of the effects of the specific 5-HT_3 agonists -PBG (A), m-CPBG (B) and 2-CH_3-5-HT (C) on isotonically suspended auricle of *Sepia officinalis*. Values are shown as the mean ± S.E.M.

through IP_3-sensitive channels after activation of the 5-HT_2 receptor-dependent PI-response. Investigations on *Venus mercenaria* tissue also revealed the same 5-HT effect (Greenberg 1960a, b). In particular, an increase in the intracellular Ca^{2+} concentration caused by α-CH_3-5-HT treatment was reported in studies on eggs fixed in the meiotic prophase from the bivalve *Ruditapes philippinarum* (Fong et al. 1997). The positive effects on tone measured at high concentrations of α-CH_3-5-HT could be explained as resulting from increasing protein kinase C (PKC) activation through rising levels of DAG and its interaction with PKC. Investigations on the cephalic aorta of *S. officinalis* with the PKC-activating substance phorbol-12,13-diacetat have already shown, that it has a stronger positive effect on tone than do noradrenaline and dopamine (Schipp, pers. commun.). Thus, the restraining effect of ketanserin can be seen in the specific interaction of α-CH_3-5-HT with a 5-HT_2-like receptor subtype in *Sepia* tissue. To conduct a more detailed investigation on enzymes putatively involved in the 5-HT_2 cell signal transduction pathway, the specific enzyme inhibitors U-73122 and 2-APB seemed to be good tools, since the presence of PLC within tissues of molluscs and especially in *S. officinalis* had previously been ascertained (Rack et al. 1992, Suzuki et al. 1999, Tierney 2001). Moreover, PLC activated by a 5-HT receptor was described in the brain of *Aplysia californica* (Li et al. 1995). Also 2-APB was described as a modulating substance of IP_3-dependent mechanisms (Ascher-Landsberg et al. 1999, Kukkonen et al. 2001). An increase in intracellular inositol-phosphates was observed with cloning experiments of a 5-HT receptor from *L. stagnalis* and its expression in eukaryotic HEK cells followed by 5-HT treatment (Gerhardt et al. 1996). Studies with U-73122 revealed similar effects on Straub-cannula preparations of *Sepia* ventricle (Versen et al. 1999). Furthermore, biochemical results proved the PI transduction mechanisms for the systemic heart of *S. officinalis* (Versen 1999). The results in the present study show that there are similar signal transduction mechanisms involved, and the membrane-permeable enzyme inhibitors mainly showed the strongest inhibitory action on the auricular contraction force. With these findings, it seems that the activation of the 5-HT_2 receptor assumed for the auricle of *S. officinalis* is also triggered via the PLC and the PI-response. For the investigation of 5-HT_3 binding sites in *Sepia* tissue, the specific 5-HT_3 agonists 1-phenylbiguanid, m-chlorphenylbiguanid and 2-CH_3-5-HT, that have been described as classical agonists acting on 5-HT_3 receptors in vertebrates, were used (Fozard 1984, Hoyer et al. 1994, Jackson and Yakel 1995). Also studies on molluscan tissues have indicated the presence of 5-HT_3-like receptors (Murakami et al. 1992, Walcourt-Ambakederemo and Winlow 1995, Green et al. 1996). Our results about the distinct actions of 5-HT_3 agonists on the auricle of *S. officinalis* indicate that mechanisms based on 5-HT_3 sensitive binding sites may be involved. The results point to different cell responses caused by 5-HT_3 agonists consisting of a tone increase with 1-PBG treatment and an increase in the contraction force only with 2-CH_3-5-HT treatment. In summary, the present study suggests that except for the 5-HT_2-like receptor, 5-HT_3-like binding sites are also present in *S. officinalis*.

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