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Serotonin Increases Calcium Current in Human Atrial Myocytes via the Newly Described 5-Hydroxytryptamine$_4$ Receptors

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SUMMARY

In various species, including humans, 5-hydroxytryptamine (5-HT) has been shown to exert positive chronotropic and inotropic cardiac effects through different types of receptors. The goal of the present study was to investigate the regulation by 5-HT of voltage-gated Ca^{2+} channels in human atrial myocytes and to characterize the receptor involved. Cardiomyocytes isolated enzymatically and mechanically were voltage-clamped using the whole-cell configuration of the patch-clamp technique. Extracellular perfusion of 5-HT increased Ca^{2+} current (I_{Ca}) amplitude with a EC_{50} (0.1 μM) similar to that observed with isoprenaline. The effects of 5-HT were blocked by the addition of protein kinase A inhibitor in the pipette. In addition, the effects of 5-HT, isoprenaline, and intracellular cAMP on I_{Ca} were not additive. These results support the hypothesis that the inotropic effect of 5-HT in human atrial myocytes is related to an increase of I_{Ca} via an elevation of intracellular cAMP levels and stimulation of cAMP-dependent protein kinase. The effects of 5-HT were not blocked by antagonists of 5-HT$_1$ (methiothepin), 5-HT$_2$ (ketanserin), or 5-HT$_3$ (ICS 205-930 at a low concentration) receptors. The benzamide derivatives renzapride and zacopride and the azabicyclobenzimidazolone derivative BIMU 8 increased I_{Ca}, but less efficiently than did 5-HT or 5-methoxytryptamine. Moreover, ICS 205-930 at high concentrations (>1 μM) completely antagonized the effects of 5-HT. Thus, the pharmacology of the 5-HT receptor involved in an increase of I_{Ca} in human atrial myocytes resembles that recently described for the 5-HT$_4$ receptor.
In atrial myocytes dissociated from rat, rabbit, guinea pig, or frog, 5-HT at high concentrations had no effect on Ca^{2+} currents, suggesting that the distribution of 5-HT$_4$ receptors in cardiac tissues is species dependent.

The effects of serotonin on heart function have been reported to be mediated directly by myocardial 5-HT$_1$-like (1, 2), 5-HT$_2$ (3), or unknown receptors (4–6). Alternatively, indirect effects have also been reported and probably involved 5-HT$_1$-like (7) or 5-HT$_3$ (8, 9) receptors located on nerve endings. Myocardial 5-HT receptors differ among species and among different regions of heart within the same species. Apart from this apparent heterogeneity in myocardial 5-HT receptors, little is known about the cellular mechanisms of the effects of 5-HT. It has been proposed that serotonin is synthesized and stored in the human heart, thus constituting a potential endogenous source of inotropic support for the failing myocardium (10). Recently, Kaumann *et al.* (11) reported that 5-HT increases contractile force in human atrial strips via a new type of 5-HT receptor, similar to the 5-HT$_4$ receptors. These sites are positively coupled to adenylate cyclase and have been described in primary cultures of fetal mice colliculi neurons (12–14). 5-HT$_4$ receptors have also been found in adult guinea pig hippocampus (15), ileum (16), and colon (17), as well as rat esophagus (18).

The voltage-sensitive Ca^{2+} current plays a central role in the regulation of cardiac excitability and contractility (19, 20). It is well documented that β-adrenergic receptor activation increases the amplitude of I_{Ca} in myocardial tissues by a mechanism involving stimulation of adenylate cyclase, increased production of cAMP, and activation of cAMP-dependent protein kinase (21, 22).

In the present study we report, for the first time, that 5-HT increases L-type Ca^{2+} channel current in human cardiac cells. We demonstrate that the increase of I_{Ca} by 5-HT is mediated by an elevation of intracellular cAMP similar to that of β-adrenergic stimulation. This effect appears to be mediated via a 5-HT$_4$ receptor and is probably responsible for the positive inotropic effect induced by 5-HT in human atrial strips (11). A preliminary report of these results has appeared elsewhere (23).

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TEA, tetraethylammonium chloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; $(Na)_2CP$, phosphocreatine (disodium salt); 5-MeOT, 5-methoxytryptamine; 2-CH_3-5-HT, 2-methyl-5-hydroxytryptamine; renzapride, (±)-*endo*-2-methoxy-4-amino-5-chloro-*N*-(1-azabicyclo-[3.3.1]-non-4-yl)benzamide monohydrochloride; BIMU 8, *N*-(*endo*-8-methyl-8-azabicyclo[3.3.1]-2,3-dihydro-3-isopropyl-2-oxo-1*H*-benzimidazol-1-carboxamide)hydrochloride; zacopride, 4-amino-*N*-(1-azabicyclo[2.2.2]oct-3-yl)-5-chloro-2-methoxybenzamide HCl; ICS 205-930, 3-α-tropanyl-1-indol-3-carboxylic acid ester; EC_{50}, half-maximal efficacy; A-PKi, protein kinase A inhibitor; I_{Ca}, calcium current.

Materials and Methods

Electrophysiology. Briefly, human atrial cells were enzymatically dissociated, by collagenase treatment, from fragments (0.5–1 cm^3) of the right atrial appendage obtained during open heart surgery on 32 patients (ages between 35 and 77 years). The clinical diagnosis was aortic and/or mitral stenosis and coronary artery disease (for complete description, see Ref. 24). None of these patients had been pretreated with Ca^{2+} channel antagonists or β-adrenergic blockers. Other medications were stopped 24 hr before surgery. Cells were allowed to settle in Petri dishes placed at the opening of a 250-μm-inner diameter capillary for extracellular perfusions. The whole-cell variation of the patch technique (25) was used. The voltage-clamp circuit was provided by a Biologic (model RK-300; Grenoble, France) patch-clamp amplifier. Recording pipettes were fire-polished before use (tip diameters, 1–2 μm; resistances, 2–4 MΩ when filled with recording solutions). After formation of a tight seal between the recording electrode and the myocyte membrane, the electrode capacitance was compensated electronically before a whole-cell recording was obtained; seal resistance were >1 GΩ. After membrane rupture, series resistances (1.5–2 times the pipette resistance) estimated from the decay of the capacitive transients were compensated (by 80–90%). Because current amplitudes were <2 nA, voltage errors resulting from the uncompensated series resistance were always <4 mV. Ca^{2+} currents from single human atrial cells were recorded by 200-msec voltage-clamp pulses to −10 mV, from an holding potential of −80 mV. The net calcium current was measured as the difference between maximal peak inward current and zero-current base line. Inactivation curves were measured after 1-sec conditioning pulses, followed by a 10-msec return to the holding potential, before measurement of currents during a positive test pulse. Contaminating K^+ and Na^+ currents were eliminated by, respectively, substitution of K^+ ions by Cs^+ in the patch pipette and of Na^+ ions by TEA^+ ions in the extracellular medium. Under these conditions, the current elicited was an inward L-type Ca^{2+} current that could be completely blocked by 1 μM nifedipine (24) and 1 mM cobalt (24). All electrophysiological experiments were performed at room temperature (22 ± 1°).

Solutions. The external solution contained (in mM) 136 TEA, 1.1 $MgCl_2$, 2 $CaCl_2$, 22 dextrose, 10.8 KCl, 25 HEPES, 10 glutamate, 0.16 penicillin, 0.068 streptomycin, and 0.05 phenol red, at pH 7.4 ± 0.1 (adjusted with KOH). Whole-cell pipette solution contained (in mM) 120 CsCl, 10 HEPES, 10 EGTA, 3 Mg-ATP, 1 Li-GTP, 5 Na_2CP, and 2 $MgCl_2$, at pH 7.3 ± 0.1 (adjusted with CsOH).

Drugs. A-PKi, 5-HT, 5-MeOT, (±)-propranolol, and isoprenaline (Sigma Company, La Verpillière Cedex, France), renzapride (Beecham Pharmaceuticals, Harlow, UK), BIMU 8 (Institut De Angeli Boehringer Ingelheim, Milan, Italy), and zacopride (Laboratoires Delalande, Rueil-Malmaison, France) were prepared as 10 mM stock solutions in distilled water. Methiothepin (Hoffman-La Roche, Basel, Switzerland) and ketanserin (Jansen Pharmaceutica, Beerse, Belgium) were prepared as 10 mM stock solutions in a tartric acid solution. ICS 205-930 (Sandoz Pharma, Basel, Switzerland) was prepared as a 10 mM stock solution in anhydrous ethanol.

Statistical analysis. Data are expressed as means ± standard errors, with n being equal to the number of cells studied.

Results

Effect of 5-HT on I_{Ca}. The extracellular perfusion of 5-HT increased the amplitude of the L-type Ca^{2+} current (Fig. 1). Under the recording conditions used here (holding potential of −80 mV), 5-HT (1 μM) induced a 5-fold increase in I_{Ca} amplitude (Fig. 1A), with the time course being 1.25 ± 0.4 min (n = 10) (Fig. 1B). This potentiation of I_{Ca} by 5-HT was dose dependent. Cumulative dose-responses of 5-HT were obtained both in the absence and in the presence of a β-adrenergic blocker [1 μM (±)-propranolol], excluding the possibility of stimulation of the β-adrenergic receptor by 5-HT (Fig. 1C).

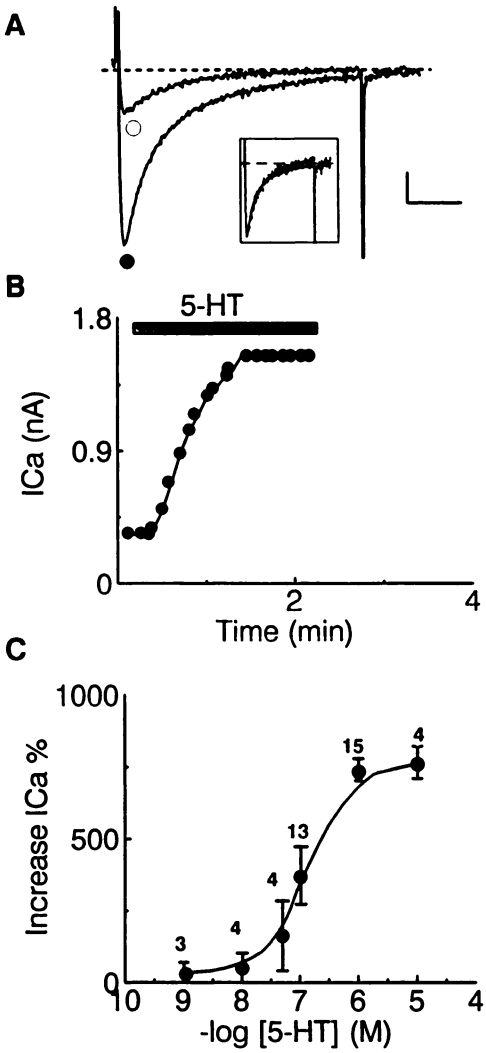

Fig. 1. Effects of 5-HT on I_{Ca}. I_{Ca} was recorded in the presence of 2 mM Ca^{2+}, TEA^+ (in the bath), and Cs^+ (in the patch pipette). A, Individual Ca^{2+} current traces evoked by a depolarizing pulse from −80 mV to −10 mV. ○, Ca^{2+} current recorded in a cell after 5 min of perfusion with the control solution, in order to obtain steady state conditions; ●, maximal increase of I_{Ca} induced by perfusion with the same solution containing 1 μM 5-HT. *Scales bars*, 0.5 nA and 40 msec. *Inset*, scaled I_{Ca} in the presence and the absence of 5-HT. B, Time course of the effect of 5-HT on I_{Ca}. The cell was perfused with 1 μM 5-HT as indicated. C, Cumulative dose-response relationship for the increase of I_{Ca} by 5-HT in the presence of 1 μM (±)-propranolol. *Points*, mean ± standard error. The number of cells investigated is indicated close to the symbols.

The threshold dose was in the submicromolar range, whereas micromolar concentrations were required for maximal effects (736 ± 28% above the control; n = 15). The concentration of 5-HT producing a half-maximal response (EC_{50}) was approximately 0.1 μM (Fig. 1C). Moreover, 5-HT (1 μM) induced a shift of both the threshold and maximum amplitudes of I_{Ca} towards negative potentials (−12 ± 4 mV; n = 10) (Fig. 2A). Steady state inactivation was measured by application of a 1-sec-duration prepulse to various depolarizing potentials before a test pulse to −10 mV (Fig. 2B). I_{Ca} inactivation began at potentials around −50 mV and reached maximal steady state inactivation at −10 mV (half-inactivation potential, −30 ± 4 mV; n = 7). A negative shift in the steady state inactivation curve of about −14 ± 7 mV (n = 7) was observed in the presence of 5-HT (Fig. 2B). The inactivation time course of I_{Ca} (biex-

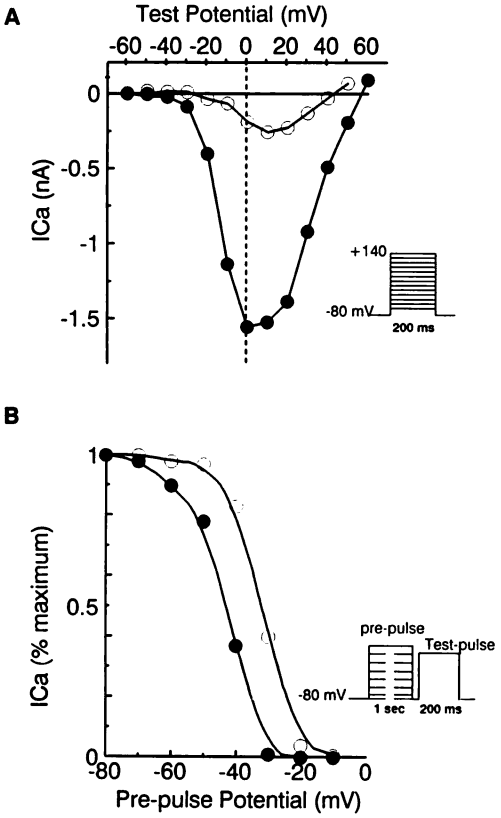

Fig. 2. Effects of 5-HT on current-voltage relationship and steady state inactivation of I_{Ca}. A, Current-voltage relationship. ○, I_{Ca} control; ●, I_{Ca} in the presence of 1 μM 5-HT. B, Steady state inactivation of I_{Ca} (measured by double-pulse protocol, which consists of the application of a 1-sec-duration prepulse to various depolarizing potentials before a test pulse to −10 mV). ○, Control; ●, effect of 1 μM 5-HT.

ponential) was not affected by 5-HT. Inactivation time constants were 15 ± 5 ms and 81 ± 14 ms for I_{Ca} control and 12 ± 2 ms and 70 ± 7 ms in the presence of 1 μM 5-HT at −10 mV (mean of 10 cells).

Evidence for a role of cAMP in mediating 5-HT effects on I_{Ca}. Perfusion of isoprenaline (1 μM) increased I_{Ca} by 800 ± 100% (n = 4) (Fig. 3A), and the maximal effect was reached after 1.08 ± 0.1 min (Fig. 3B). A negative shift in the current-potential relationship of I_{Ca} (about −10 mV) was also observed (Fig. 3C).

It was documented, in cardiac tissue, that the production of cAMP is a key point in the stimulation of cAMP-dependent protein kinase A, which subsequently phosphorylates the Ca^{2+} channel (22). Intracellular perfusion of A-PKi prevents the phosphorylation of the Ca^{2+} channel (22). Fig. 4A shows the effect of 5-HT on I_{Ca} recorded on the same batch of myocytes in the absence and in the presence of A-PKi in the pipette. In the absence of A-PKi in the pipette (Fig. 4A, *a*), the perfusion of 5-HT (10 μM) increased I_{Ca} by 724 ± 100% (n = 10). In contrast, in the presence of 10 μM A-PKi in the pipette (Fig. 4A, *b*), 5-HT increased I_{Ca} by only 47 ± 20% (n = 10). In addition, in cardiac cells, high intracellular cAMP concentrations and extracellular isoprenaline stimulate I_{Ca} in a nonadditive fashion (26, 27). We show in Fig. 4B that, when 50 μM cAMP was added in the recording pipette (Fig. 4B, *b*), 5-HT at concentrations up to 10 μM was unable to increase I_{Ca} further (Fig. 4B). Similarly, the effects of 5-HT (10 μM) and isoprenaline (10 μM) were not additive (data not shown). These data

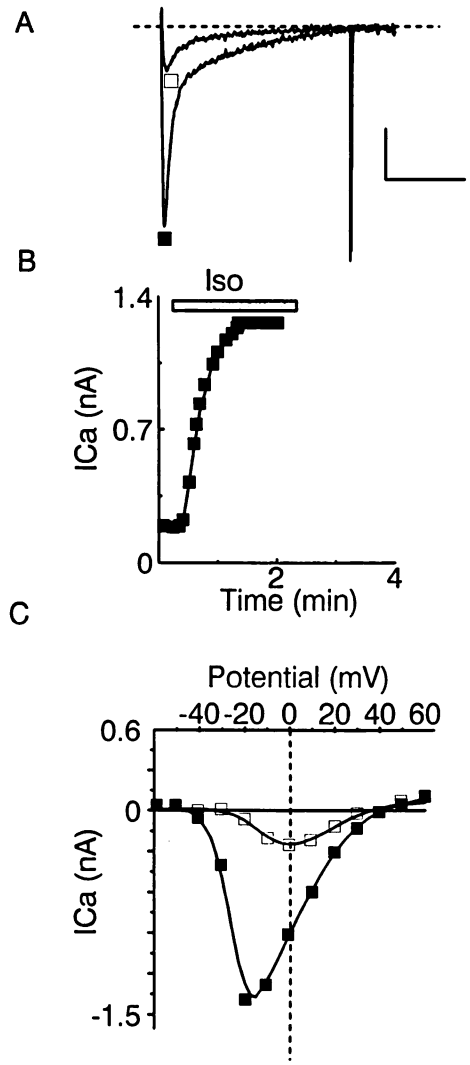

Fig. 3. Effects of isoprenaline on I_{Ca}. A, Individual Ca^{2+} current traces evoked by a depolarizing pulse from −80 mV to −10 mV. □, I_{Ca} control; ■, maximal increase of I_{Ca} by 1 μM isoprenaline. *Scales bars*, 0.5 nA and 90 msec. B, Time course of the effect of 1 μM isoprenaline (*Iso*) on I_{Ca}. C, Current-voltage relationship. □, I_{Ca} control; ■, I_{Ca} in the presence of 1 μM isoprenaline.

support a scheme in which both agonists share a common limiting cascade after receptor activation.

Taken together, all these results are consistent with the idea that the positive inotropic effect of 5-HT is mediated via a receptor that is positively coupled to adenylate cyclase.

Nature of the 5-HT receptor involved. The pharmacological characteristics of the 5-HT receptors involved in the increase of I_{Ca} by 5-HT were examined using different specific antagonists of 5-HT_1 (methiothepin), 5-HT_2 (ketanserin), and 5-HT_3 (ICS 205-930 used at a low concentration, <1 μM) receptors. All experiments were conducted in the presence of 1 μM (±)-propranolol, to eliminate a possible involvement of β receptors. I_{Ca}, once increased by 0.1 μM 5-HT, was not significantly affected by subsequent application of methiothepin (10 μM), ketanserin (10 μM), or ICS 205-930 (0.1 μM). Ca^{2+} current after application of these compounds was, respectively, 98 ± 1% (n = 4), 90 ± 1% (n = 5), and 98 ± 1% (n = 5) of the current in the presence of 5-HT (taken as 100%), a result effectively excluding any involvement of 5-HT_1, 5-HT_2, and 5-

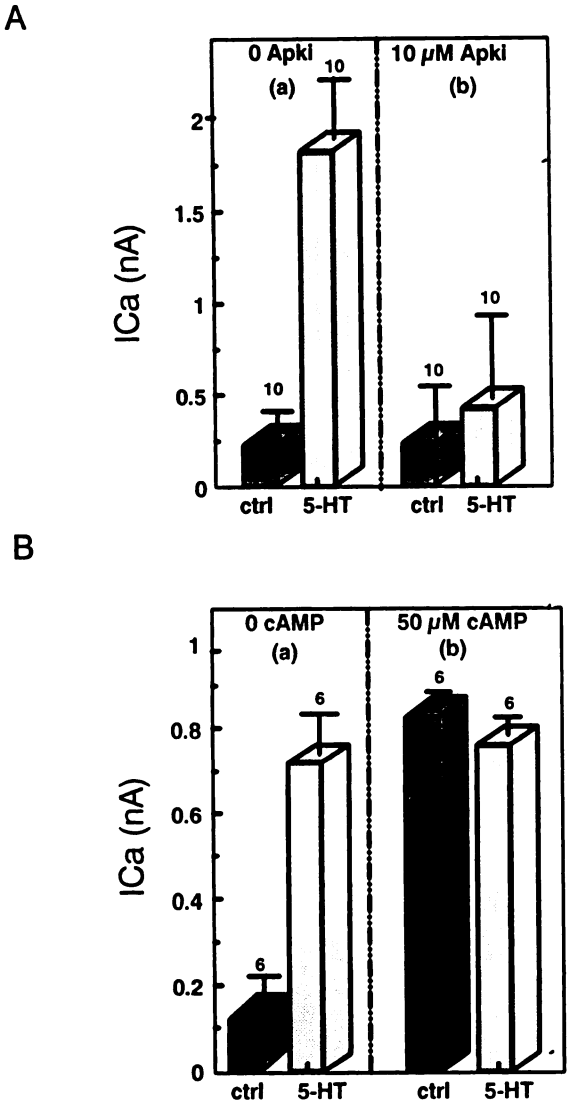

Fig. 4. Evidence for a role of cAMP in mediating 5-HT effects on I_{Ca}. Ca^{2+} current was recorded with a depolarizing pulse from −80 mV to −10 mV. A, Effect of 10 μM 5-HT on I_{Ca} recorded in the absence (*a*) or in the presence (*b*) of 10 μM A-PKi in the patch pipette. *Numbers*, number of cells. *Error bars*, standard errors. B, Effect of 10 μM 5-HT on I_{Ca} recorded without (*a*) and with (*b*) 50 μM cAMP in the pipette. *Numbers*, number of cells. *Error bars*, standard errors.

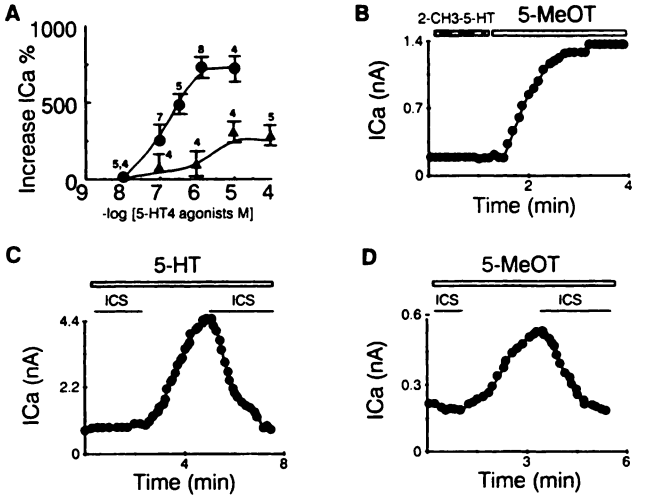

Fig. 5. Effect on I_{Ca} of extracellular perfusion of different concentrations of 5-HT_4 agonists and antagonists, in the presence of 1 μM (±)-propranolol. Ca^{2+} current was recorded with a depolarizing pulse from −80 mV to −10 mV. A, Cumulative dose-response relationship of the potentiation of I_{Ca} by 5-MeOT (●) and renzapride (▲). *Points*, mean ± standard error. The number of cells investigated is indicated close to the symbols. B, Effect of 10 μM 2-CH_3-5-HT and 1 μM 5-MeOT on I_{Ca}. C and D, ICS 205-930 (10 μM) prevents the potentiation of I_{Ca} by both 0.1 μM 5-HT (C) and 1 μM 5-MeOT (D).

HT_3 receptors in the effect described here. However, agonists of the newly described 5-HT_4 receptors, 5-MeOT (12, 16), renzapride (13, 14, 28), BIMU 8 (29, 30), and zacopride (31), were found to increase I_{Ca}. The responses to 5-MeOT and renzapride were dose dependent (Fig. 5A), and the half-maximal effects were obtained at 0.3 and 3 μM, respectively. The increases of I_{Ca} by 5-MeOT (1 μM), renzapride (1 μM), BIMU 8 (10 μM), and zacopride (1 μM) were, respectively, 749 ± 16% (n = 8) (see also Fig. 5B), 77 ± 47% (n = 4), 113 ± 50% (n = 6), and 106 ± 40% (n = 4). Moreover, 2-CH_3-5-HT (10 μM), which is a 5-HT_3 agonist, was without effect on I_{Ca} (Fig. 5B).

Previous studies have reported that nanomolar concentrations of ICS 205-930 block 5-HT_3 receptors (9), whereas at high concentrations it acts as a 5-HT_4 receptor antagonist (11, 12, 17, 32). Effects of higher concentrations of ICS 205-930 (10 μM) on I_{Ca} were tested in the presence of 5-HT and 5-MeOT (Fig. 5). Perfusion with 10 μM ICS 250-930 completely antagonized I_{Ca} stimulation by 0.1 μM 5-HT (Fig. 5C) or 1 μM 5-MeOT (Fig. 5D). Similar results were found with 1 μM renzapride, 1 μM BIMU 8, and 1 μM zacopride (data not shown). ICS 205-930 (10 μM) was without effect on isoprenaline-stimulated I_{Ca} (data not shown).

Species dependence of 5-HT effects on I_{Ca}. We have compared the effect of 5-HT and isoprenaline on myocytes isolated with the same procedure from rat, guinea pig, rabbit, and frog atria. Whereas 1 μM isoprenaline stimulated I_{Ca} in all preparations, 5-HT (10 μM) neither affected I_{Ca} in any of these cells (Fig. 6) nor increased the contractile force on atrial strips (data not shown) in any of these species.

Discussion

The most important result of this report is that 5-HT, acting on 5-HT_4 receptors, is as potent as isoprenaline in increasing I_{Ca} in human atrial myocytes. In cardiac tissues, it is well documented that β-adrenergic agonist-induced activation of I_{Ca} is mediated by an increase in cAMP levels, a stimulation of cAMP-dependent protein kinase, and a phosphorylation of L-type Ca^{2+} channels (22, 26). Several lines of evidence presented here indicate that the mechanisms of action of 5-HT in increasing I_{Ca} are similar to those of β-adrenergic receptors. (i) The stimulatory effects of isoprenaline and 5-HT on I_{Ca} were not additive. (ii) Both agonists produced a shift toward negative potentials (−10 mV) of the current-potential and of the steady state inactivation relationship for I_{Ca}. (iii) When saturating concentrations of A-PKi or cAMP were added inside of the patch pipette, no increase of I_{Ca} was induced by 5-HT.

Taken together, these results strongly indicate that both 5-HT and β-adrenergic agonists increase I_{Ca} through the same cAMP-dependent intracellular pathway. Our results are in agreement with the findings of Kaumann *et al.* (11), who reported that 5-HT (10 μM) increased cAMP levels and stim-

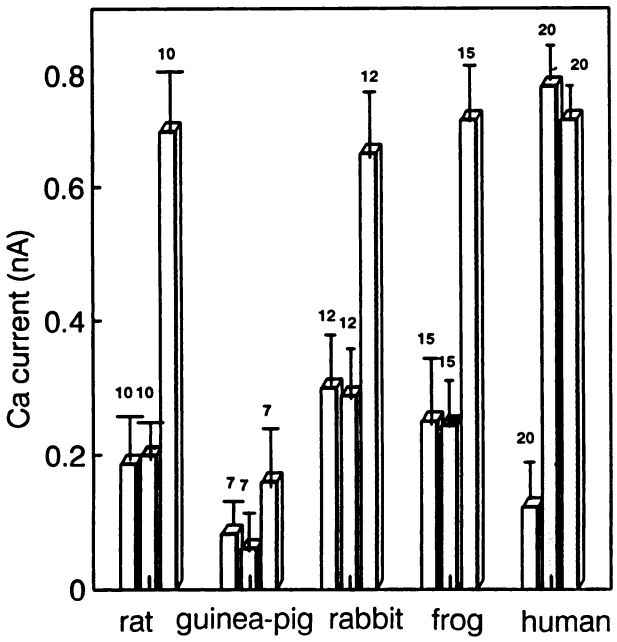

Fig. 6. Comparison of the effects of 5-HT and isoprenaline on atrial myocytes isolated from rat, guinea pig, rabbit, frog, or human. Ca^{2+} current was recorded with a depolarizing pulse from −80 mV to −10 mV. □, I_{Ca} control; ■, effect of 10 μM 5-HT; ▩, effect of 10 μM isoprenaline in the presence of 10 μM 5-HT. *Numbers*, number of cells. *Error bars*, standard errors.

ulated cAMP-dependent protein kinase activity in human atria strips.

The stimulatory effects of 5-HT on cAMP levels in human atrial strips have been demonstrated to be mediated by activation of the newly described 5-HT_4 receptors (11). Similarly, we demonstrate here that 5-HT stimulatory effects on I_{Ca} were mediated by 5-HT_4 receptors. (i) None of the 5-HT_1, 5-HT_2, or 5-HT_3 antagonists used were able to inhibit the 5-HT effects on I_{Ca}. (ii) 5-MeOT was a full potent agonist of the 5-HT receptors involved in the increase of I_{Ca}. 5-MeOT has also been described as a full potent agonist on 5-HT_4 receptors in guinea pig brain (15), ileum (16), and colon (17). In contrast, 5-MeOT is inactive at 5-HT_3 receptors (16), excluding their implication in 5-HT effects on I_{Ca}. (iii) Benzamide (renzapride, zacopride) and azabicyclobenzimidazolone (BIMU 8) derivatives were also able to increase I_{Ca}. These two classes of drugs have been shown to be agonists at 5-HT_4 receptors. However, we found that benzamides and azabicyclobenzimidazolone were weak partial agonists of the 5-HT_4 receptors in human atrial myocytes. In fact, benzamides are full agonists in colliculi neurons (12, 14, 29) and guinea pig ileum (32), partial agonists in guinea pig ascending colon (17), rat esophagus (18), and guinea pig hippocampus (15), and even antagonists in rat hippocampal CA1 neurons (33). It is difficult at this stage to know whether these differences reflect small differences in the structure of the 5-HT_4 receptors present in these different organs or differences in the transduction mechanisms. (iv) ICS 205-930 at high concentrations (10 μM) was able to block 5-HT-, 5-MeOT-, renzapride-, BIMU 8-, and zacopride-induced increases in I_{Ca}. ICS 205-930 at high doses (>1 μM) is the only compound, so far, that has been found to block 5-HT_4-mediated receptor effects whatever the cellular model studied. This report is the first demonstration that 5-HT_4 receptors in human atria are directly localized on myocytes.

An interesting finding shown in the present study is that 5-HT_4 receptors are able to increase I_{Ca} in human atrial myocytes but not in atrial myocytes of several other species (rabbit, guinea pig, rat, or frog). Possible explanations for this differences are (i) species-dependent distribution of 5-HT_4 receptors, (ii) age-dependent expression of 5-HT_4 receptors (atrial cells only from patients between 35 and 77 years of age have been used), (iii) pathology- and/or treatment-induced *de novo* expression of 5-HT_4 receptors, or (iv) revelation of silent 5-HT_4 receptors by pathogenesis and/or treatment. However, it should be noted that 5-HT_4 receptors were also expressed by the patients who had been pretreated with Ca^{2+} channel antagonists or β-adrenergic blockers.

In summary, 5-HT increases L-type Ca^{2+} channel current in human cardiac cells. The increase of I_{Ca} by 5-HT involves an elevation of intracellular cAMP likely to promote a phosphorylation of the Ca^{2+} channel. Quantitative pharmacological analyses suggest that the receptor is distinct from the 5-HT_1, 5-HT_2, and 5-HT_3 subtypes. The pharmacological profile of the 5-HT receptor localized on the myocytes exhibits considerable similarities to that of the recently described 5-HT_4 receptor. The presence, on human atrial myocytes, of 5-HT_4 receptors that have a potent positive inotropic effect may offer new perspectives in the development of therapeutic cardiotonic drugs.

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