Effect of 5-HT₄ receptor stimulation on the pacemaker current $I_f$ in human isolated atrial myocytes

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Abstract

Objective: 5-HT receptors are present in human atrial cells and their stimulation has been implicated in the genesis of atrial arrhythmias including atrial fibrillation. An $I_f$-like current has been recorded in human atrial myocytes, where it is modulated by $\beta$-adrenergic stimulation. In the present study, we investigated the effect of serotonin (5-hydroxytryptamine, 5-HT) on $I_f$ electrophysiological properties, in order to get an insight into the possible contribution of $I_f$ to the arrhythmogenic action of 5-HT in human atria.

Methods: Human atrial myocytes were isolated by enzymatic digestion from samples of atrial appendage of patients undergoing corrective cardiac surgery. Patch-clamped cells were superfused with a modified Tyrode's solution in order to amplify $I_f$ and reduce overlapping currents.

Results and conclusions: A time-dependent, cesium-sensitive increasing inward current, that we had previously described having the electrophysiological properties of the pacemaker current $I_f$, was elicited by negative steps ($-60$ to $-130$ mV) from a holding potential of $-40$ mV. Boltzmann fit of control activation curves gave a midpoint ($V_{1/2}$) of $-88.9\pm2.6$ mV ($n=14$). 5-HT (1 $\mu$M) consistently caused a positive shift of $V_{1/2}$ of $11.0\pm2.0$ mV ($n=8$, $p<0.001$) of the activation curve toward less negative potentials, thus increasing the amount of current activated by clamp steps near the physiological maximum diastolic potential of these cells. The effect was dose-dependent, the EC₅₀ being 0.14 $\mu$M. Maximum current amplitude was not changed by 5-HT. 5-HT did not increase $I_f$ amplitude when the current was maximally activated by cAMP perfused into the cell. The selective 5-HT₄ antagonists, DAU 6285 (10 $\mu$M) and GR 125487 (1 $\mu$M), completely prevented the effect of 5-HT on $I_f$. The shift of $V_{1/2}$ caused by 1 $\mu$M 5-HT in the presence of DAU 6285 or GR 125487 was $0.3\pm1$ mV ($n=6$) and $1.0\pm0.6$ mV ($n=5$), respectively ($p<0.01$ versus 5-HT alone). The effect of 5-HT₄ receptor blockade was specific, since neither DAU 6285 nor GR 125487 prevented the effect of 1 $\mu$M isoprenaline on $I_f$. Thus, 5-HT₄ stimulation increases $I_f$ in human atrial myocytes; this effect may contribute to the arrhythmogenic action of 5-HT in the human atrium.

Keywords: Human atrial myocytes; Serotonin (5-HT); 5-HT₄ receptor subtype; Arrhythmia (mechanisms); Pacemaker current

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) exerts multiple effects on the cardiovascular system [1]. In the human and pig atrium, 5-HT causes positive chronotropic, inotropic, and lusitropic effects [2,3], through stimulation of the 5-HT₄ receptor subtype. The human atrial 5-HT₄ receptor has been recently cloned; it appears to be different from the 5-HT₃ receptor isoform present in the brain and gastrointestinal tract [4]. Stimulation of the 5-HT₄ receptor is associated with the increase in cAMP levels and activation of cAMP-dependent protein kinase [2,3,5]. One of the consequences is the 5-HT₄ receptor-mediated increase of L-type calcium current ($I_{Ca,L}$) which has been demonstrated in isolated human atrial cardiomyocytes [6].
This effect is similar to that exerted by ß-adrenoceptor stimulation [6], and is the basis for the positive inotropic response to 5-HT. This positive inotropic effect is, however, observed in the human atrium, but not in the human ventricle [7]. Furthermore, the cardiac responses to 5-HT appear to be present only in few mammalian species (human, pig, monkey) and absent in common laboratory animals, such as the rat, guinea pig and rabbit [2,6].

Due to its unique location in the human atrium, it has been suggested that 5-HT receptors may be implicated in the control of atrial activity [8]. Since 5-HT has been reported to cause arrhythmias in the isolated human atrium [9], the suggestion that 5-HT released by platelets may be involved in the genesis of atrial arrhythmias and particularly of atrial fibrillation [8], appears particularly appealing. The mechanism for induction of the arrhythmia could be an intracellular calcium overload resulting in delayed afterdepolarizations and triggered activity, as consequence of the activation of the cAMP-dependent cascade induced by 5-HT through the stimulation of 5-HT\(_4\) receptors and causing an increased calcium entry through L-type calcium channels [8].

We recently reported that the pacemaker current \(I_\text{f}\) is present in 82% of atrial myocytes isolated from the human right appendage of patients undergoing cardiac surgery [10]. The half maximal activation voltage \((V_{1/2})\) of \(I_\text{f}\) was around −86 mV which does not support a functional role for this current under physiological conditions in human atrial myocytes. In fact, the maximum diastolic potential in human atrial myocytes has been reported to be generally below this value (i.e., −70/−75 mV) [11–16], implying that the current undergoes little, if any, activation at these potentials. There are at least two ways by which such a current could become functionally important. First, its properties are modified by disease (e.g. atrial dilatation), similarly to what is observed for other ionic currents during hypertrophy or failure [17–22], or chronic atrial fibrillation [23], in such a way that it may become active under pathological conditions. A second possibility is that \(I_\text{f}\) activation curve is shifted toward less negative (and more physiological) potentials by the stimulation of relevant receptors. Recently, it was found that the \(V_{1/2}\) of \(I_\text{f}\) in human atrial myocytes was significantly shifted toward less negative potentials by ß-adrenoceptor stimulation with isoprenaline [10]. In rabbit sino-atrial node cells it has been clearly demonstrated that the effect of cAMP on \(I_\text{f}\) is largely direct and does not involve the phosphorylation of the channel [24,25]. It is likely that a similar mechanism is operative in the human heart; direct proof of the mechanism by which cAMP regulates \(I_\text{f}\) in the human atria is not available.

To get an insight into the possible contribution of \(I_\text{f}\) to the arrhythmogenic action of 5-HT in human atria, we thought that it would be important to know if 5-HT, through the stimulation of 5-HT\(_4\) receptors, was able to affect \(I_\text{f}\) electrophysiological properties (amplitude and activation voltage), and if its effects on \(I_\text{f}\) were observed at the concentrations previously demonstrated to increase \(I_{\text{Ca,L}}\).

Preliminary data have been presented in abstract form [26].

2. Methods

2.1. Cell isolation and storage

The procedure used was similar to that described previously [10]. Small samples from human right atrial appendages were used to isolate single myocytes. The specimens, obtained from the tissue routinely excised during the preparation of extra-corporeal circulation, were brought to the laboratory in cold transport saline (see Section 2.4). In the laboratory the sample was placed in Ca\(^{2+}\)-free oxygen-saturated solution. Epicardial fat and connective tissue were removed from the muscle specimen. The tissue samples were cut into small pieces of about 1 mm\(^3\) in size and gently stirred in a thermostated (35°C) Ca\(^{2+}\)-free oxygen-saturated solution for 20 min in a tissue dissociation vessel [27,28]. Cells were isolated by enzymatic dissociation in Ca\(^{2+}\)-free solution plus collagenase type V (350 I.U. ml\(^{-1}\), Sigma) and protease type XXIV (4 I.U. ml\(^{-1}\), Sigma) for 20 min. After this step was completed, the samples were stirred for 20 min in a Ca\(^{2+}\)-free solution containing collagenase type V (350 I.U. ml\(^{-1}\)). The solution was then replaced by a new enzymatic solution where the collagenase concentration was reduced to 175 I.U. ml\(^{-1}\). During this final step, the supernatant was replaced by fresh collagenase solution every 15 min and the dissociation was followed by microscopic examination of the collected medium. Cells were then stored in a Kraft–Brühe solution and allowed to sediment under gravity for 15 min or with gentle centrifugation (5 min at 1000 rpm). The supernatant was removed and replaced by Ca\(^{2+}\)-free Tyrode’s solution. The Ca\(^{2+}\) concentration was raised stepwise (50, 100, 200, 500 μM). The isolated cells were kept at room temperature in Tyrode’s solution containing penicillin (50 I.U. ml\(^{-1}\)) and streptomycin (50 I.U. ml\(^{-1}\)) and used within the day.

2.2. Patients

Tissue specimens were obtained from 18 patients of both sexes (age: 67±10 years, mean±S.D.) undergoing cardiac surgery, for cardiac vascular disease (67%) or aortic valve replacement (33%). All patients were in sinus rhythm; they were under treatment with various combinations of drugs, including Ca\(^{2+}\)-antagonists, acetylsalicylic acid and nitrate compounds. Our previous results [10] suggested that the properties of \(I_\text{f}\) were not influenced by the underlying pathology or pharmacological treatment in
any evident manner. However, patients chronically treated with β-adrenergic blockers were excluded due to the reported sensitization of human atrial 5-HT₄ receptors induced by such therapy [29]. All patients gave their informed consent for the use of their tissue samples. The design of this study conforms to the Helsinki declaration [30] and was approved by the local ethical committee.

2.3. Electrophysiological recordings

The patch clamp technique (whole cell recording) was used to measure the electrophysiological properties of the isolated human atrial myocytes. Details of the equipment have been previously described [10]. Cells were placed in an experimental bath on the platform of an inverted microscope (Nikon Diaphot TMD, Japan). Experiments were performed using a patch amplifier (Axopatch-1B, Axon Instruments, CA, USA) interfaced to a personal computer by means of a general purpose DAC/ADC interface (Labmaster Tekmar, Scientific Solutions). Data were viewed on-line on an analogic oscilloscope and on the computer screen. Data acquisition and preliminary analysis were performed by means of the integrated software package pClamp (Axon Instruments). Cells were superfused with a normal Tyrode’s solution (see Section 2.4); a modified Tyrode’s solution was used during measurements of the hyperpolarization-activated inward current (Iₜ). Temperature was maintained in the range 36–37°C. Patch-clamp pipettes were prepared from glass capillary tubes (Garner, CA, USA) by means of a two-stage vertical puller (Hans Otchoski, Hamburg, Germany) and fire-polished just before use. Pipettes had a resistance of 1.5–2.5 MΩ when filled with the internal solution.

For the experiments with drug application, the patch-clamped cell was superfused by means of a temperature-controlled micro-superfusor which allowed rapid changes of the solution bathing the cell; this enabled us to minimize current run-down and to reduce exposure of the other cells present in the experimental chamber to drugs. 5-HT was dissolved in distilled water to get a stock solution with a final concentration of 10 mM containing 1 mg/ml ascorbic acid. The stock solution was then diluted with Tyrode’s solution to get the final 5-HT concentrations.

The selective 5-HT₄ antagonists, DAU 6285 (N-endo-8-methyl-8-azabicyclo[3.2.1]-oct-3-yl)-2,3-dihydro-2-oxo-1H-benzimidazole-1-carboxamide,hydrochloride) [31], and GR 125487 [(1R-[2-(methylsulphonylamino)-ethyl]-4-piperidinyl]-methyl-1H-indole-3-carboxylate) [3-233], were dissolved in distilled water to get the stock solutions (both 10 mM) and then diluted in Tyrode’s solution up to the final concentrations. In the experiments aimed to test the role of cAMP in mediating 5-HT effect, the tip of the patch pipette was filled with the internal solution and then backfilled with the same solution also containing 200 μM cAMP (Sigma).

2.4. Solutions

The composition of the solutions employed was as follows (in mM): Transport saline: Lactic acid 286; NaCl 103; KCl 5.4; CaCl₂ 1.8; pH adjusted to 7.0 with NaOH. Isolation solution: NaCl 120, KCl 10, KH₂PO₄ 1.2, MgCl₂ 1.2, d- (+)-glucose 10, Heps 10, taurine 20; pH adjusted to 7.2 with NaOH. Kraft-Brühe solution: KCl 85; KH₂PO₄ 30; MgSO₄ 5.0; glucose 20; pyruvic acid 5.0; creatine 5.0; taurine 5.0; EGTA 0.5; β-hydroxybutyric acid 5.0; succinic acid 5.0; K₂-ATP 2.0; pH adjusted to 7.0 with KOH. Normal Tyrode’s solution: NaCl, 140; KCl, 5.4; CaCl₂, 1.0; MgCl₂, 1.2; Heps-NaOH pH 7.35, 5.0; glucose, 10. Modified Tyrode’s solution for hyperpolarization-activated inward current measurements: NaCl, 140; KCl 25; CaCl₂, 1.0; MgCl₂, 1.2; Heps-NaOH pH 7.35, 5.0; glucose, 10; BaCl₂ 1.0 (to block inward rectifier-like channels); MnCl₂ 2.0 (to block Iₜ,c); 4-aminopyridine 0.5 (to block transient outward potassium current, Iₒₒ). Internal solution (used to fill the patch micro-pipettes): KCl 110, ATP/K⁺ 4.3, MgCl₂ 2.0, CaCl₂ 1.0, EGTA 11, Heps/K⁺ 10, adjusted to pH 7.4 with KOH (free [Ca²⁺] < 10⁻⁷ M).

2.5. Data analysis and statistics

Data analysis and fitting were performed by using the ORIGIN 4.1 program (MicroCal, MA, USA) running on a Pentium personal computer. For fitting functions, non-linear models of convergence to solutions were used.

To evaluate steady-state values of the hyperpolarization-activated current, data were fitted to an exponential decay, which gave the time constant of Iₜ activation (τ). The difference between the extrapolated value and that of the current at the end of the hyperpolarization step was usually small (in the order of a few pA).

A Boltzmann model based on the partition theorem according to the general equation:

$$y = \frac{1}{1 + e^{(V - V_{1/2})/k}}$$

was fitted to the activation data, where V (mV) is the test membrane potential, V_{1/2} (mV) is the fitted potential for half activation and k (mV) is related to the slope of the activation curve.

Cell membrane capacitance (Cₘ) was measured by applying a pulse of small amplitude (ΔV = 10 mV) starting from a holding potential of −70 mV. The current transient following this clamp protocol was fitted with a monoeXponential model to compute series resistance (Rₛ) and then Cₘ using the two equations given below:

$$R_s = \frac{\Delta V_m}{I_{\text{peak}} \cdot 10^4}$$

and
\[ C_m = \frac{\tau}{R_i} \]

where \( I_{\text{peak}} \) is the maximum level of current (relative to the holding current) following the depolarization and \( \tau \) is the time constant of the exponential current decay. The membrane capacitance values obtained have been used to compute ionic current densities (\( I_x \) density in pA/pF).

Dose-effect curves were fitted by using the Hill equation:

\[ y = E_{\text{max}} \times \frac{x}{k + x} \]

where \( E_{\text{max}} \) is the maximum effect, \( k \) corresponds to the concentration at which 50% \( E_{\text{max}} \) is obtained (EC\(_{50}\)) and \( x \) is the concentration of drug.

Given the relatively small number of observations and the nature of the measured data that are likely to be non-normally distributed, non-parametric statistics (Wilcoxon) on paired data (one- or two-tail, according to the need) were used. All results are reported as means±S.E.M.; \( p < 0.05 \) was considered significant.

3. Results

Cells isolated from the right atrial appendages of human hearts were relatively small, with an average membrane capacitance (\( C_m \)) of 62.1±3.6 pF (\( n = 112 \)). These data are consistent with the previously reported values for capacitance and geometrical dimensions of the cells [10]. As expected, following clamp steps to hyperpolarized membrane potentials, a time-dependent inward current was present in about 80% of the cells; since we have previously documented that this current has the electrophysiological properties of \( I_t \) [10], we will call it \( I_t \).

Fig. 1 (panel A) shows the hyperpolarization-activated current (\( I_h \)) recorded from a human atrial myocyte bathed in the modified Tyrode’s solution used to amplify \( I_t \) [10]. The current traces were evoked by steps at potentials in the range of the diastolic potential of normally polarized atrial myocytes. After superfusion with 1 \( \mu \text{M 5-HT} \) (panel B), the amplitude of the current at each potential was clearly increased. The activation curve obtained by plotting current amplitude vs. membrane potential was fitted by a Boltzmann function whose average midpoint was \(-88.9±2.6 \text{ mV} \) (\( n = 14 \)), and the average current density, measured at \(-120 \text{ mV} \), was 4.3±2.0 pA/pF. Panel C of Fig. 1 shows that superfusion with 5-HT (1 \( \mu \text{M} \)) did not affect maximum current amplitude, but shifted the activation curve of \( I_h \) toward less negative potentials. The mean maximum current amplitude was 300±46 pA in controls (\( n = 8 \)) and 275±41 pA after 5-HT (\( n = 8 \)); also the slope factor \( k \) was not significantly changed being 10.4±1.2 mV in controls and 10.1±1.3 mV in the presence of 5-HT. The shift of the activation curve caused by 5-HT was 11.0±2.0 mV (\( n = 8 \), \( p < 0.001 \)). In the presence of 5-HT the activation kinetics of \( I_h \) was consistently faster at every potential (Fig. 1, panel D).

Fig. 2 shows that the shift of the activation curve of \( I_h \) measured with different concentrations of 5-HT, was dose-dependent. For comparison the dose–response curve of the effect of 5-HT on the shift of the voltage–activation relationship for \( I_t \) (present results) is shown together with that derived from the data regarding the effects of 5-HT on \( I_{\text{C,a}} \) amplitude [6]. It is apparent that the two curves are practically superimposable, the EC\(_{50}\) for the effect on \( I_t \)
being 0.14 μM, and that for the effect on $I_{Ca,L}$ being 0.13 μM; in both cases the maximal effect of 5-HT was reached at the concentration of 1 μM. The increase in $I_{Ca,L}$ caused by 5-HT in human atria involves an increase in intracellular cAMP [6].

Fig. 3 suggests that a cAMP dependent pathway is also involved in the effect of 5-HT on $I_f$. In fact, increasing intracellular cAMP by adding it to the patch pipette at a concentration of 200 μM, causes a marked increase in $I_f$ amplitude (panel A). Panel B shows the time-course of the cAMP effect: after rupturing the seal and dialysing the intracellular milieu with cAMP, $I_f$ amplitude increases rapidly, reaching the steady-state in about 2 min (panel B). When the maximal effect is reached, i.e. after 3 min of cell dialysis, superfusion of the cell with a solution containing 5-HT (1 μM), does not cause any further increase in the current amplitude. This is clearly shown in panel A, where the current traces recorded before and after 5-HT are superimposable and in panel B which clearly demonstrates that superfusion with 5-HT (arrow) does not change the amplitude of $I_f$, which remains stable over time. Similar results were obtained in five cells. Panel C of Fig. 3 shows the current–voltage relationship obtained in the same myocyte before and after diffusion of cAMP into the cell, in the presence or in the absence of 5-HT. It is apparent that 5-HT does not exert any effect on the current–voltage curve when intracellular cAMP is increased. To demonstrate that the effect of 5-HT on $I_f$ was due to the stimulation of the 5-HT$_4$ receptor subtype, cells were exposed to the selective 5-HT$_4$ receptor antagonist DAU 6285 [34].

As shown in Fig. 4 (upper panels), exposure to DAU 6285 (10 μM) for 2 min completely prevented the effect of 5-HT on $I_f$ amplitude. $I_f$-current traces recorded before and after application of 5-HT 1 μM (in the presence of DAU 6285) during steps at −60, −70, and −80 mV were identical. The activation curves obtained in control conditions, in the presence of DAU 6285 alone and of DAU 6285 plus 5-HT were fully superimposable. A similar effect was observed with the more selective 5-HT$_4$ antagonist GR 125487 [32,33] (data not shown).

Fig. 5 summarizes the results obtained with the two 5-HT$_4$ antagonists. Both DAU 6285 and GR 125487 completely prevented the shift of the $I_f$ activation curve...
caused by 5-HT: in fact, the shift of $V_{1/2}$ caused by 1 $\mu$m 5-HT in the presence of 10 $\mu$m DAU 6285 or 1 $\mu$m GR 125487 was 0.3±1 mV ($n=6$) and 1.0±0.6 mV ($n=5$), respectively ($p<0.01$ versus 5-HT alone). The effect of 5-HT$_4$ receptor blockade was specific as demonstrated in Fig. 5. In fact, exposure of the cells to DAU 6285 did not prevent the effect of isoprenaline (ISO). A 1 $\mu$m concentration of ISO which increases $I_r$ amplitude in human atrial myocytes [10], also increased $I_r$ amplitude in the presence of the 5-HT$_4$ antagonist DAU 6285. Similar results were obtained with the 5-HT$_4$ antagonist GR 125487 (not shown).

4. Discussion

The present results demonstrate that the pacemaker current $I_r$ in human atrial myocytes is modulated by 5-HT. 5-HT increases $I_r$ amplitude through the stimulation of the 5-HT$_4$ receptor subtype and involves the activation of the cAMP pathway. The effect of 5-HT is completely prevented by the selective antagonists of the 5-HT$_4$ receptor subtype, GR 125487 and DAU 6285. These compounds have a selectivity at least 30-fold higher versus the 5-HT$_4$ receptor subtype compared to other 5-HT receptor subtypes [31,32,34]. Furthermore, it has been clearly demonstrated that the 5-HT$_4$ subtype is the only 5-HT receptor functionally present in the human atrium [4,9].

The mechanism by which 5-HT$_4$ stimulation increase $I_r$ amplitude is by shifting its activation voltage toward less negative values. The voltage of half maximal activation for $I_r$, i.e. the voltage at which the current is activated at half of its maximal value, in the human atrial cells is approximately ~90 mV. This property makes unlikely a functional contribution of $I_r$ under physiological conditions, since the maximum diastolic potential of human atrial cells is rarely so negative [11–16]. However, a shift of the activation voltage of $I_r$ toward less negative values may completely change the scenario. Let us suppose that a shift occurs, as experimentally observed with 5-HT in our study, of 11 mV toward less negative values: this means that at ~70 mV (a likely maximum diastolic potential for an atrial cell) a substantial fraction of the current (~40%) will now be activated and possibly could play a functional role, i.e. leading to the development of a diastolic depolarization phase. The effect of 5-HT is similar to that previously observed with $\beta$-adrenergic receptor stimulation [10]. This is also confirmed in the present study; in fact when 5-HT$_4$ receptors are blocked, ISO causes an increase in $I_r$ amplitude, which is indistinguishable from that caused by 5-HT. Thus, as for the effect of $\beta$-adrenergic receptor stimulation, it is likely that the effect occurs through the activation of the adenyl cyclase pathway, leading to an increase in cAMP that is the same pathway involved in the 5-HT$_4$ mediated increase in $I_{Ca,L}$ [6]. This is strongly suggested also by the observed increase of $I_r$ amplitude when the cAMP is diffused into the cell and by the lack of effect of 5-HT, when $I_r$ is maximally activated by intracellular cAMP. However the mechanisms by which cAMP regulates $I_{Ca,L}$ and $I_r$ are likely to differ. In fact the effect of cAMP on $I_r$ has been demonstrated to be largely direct and independent of channel phosphorylation [24,25]. It is likely that such a mechanism could regulates $I_r$ also in human atria, even if direct data are not available. It is worth noting that the EC$_{50}$ for the increase in $I_{Ca,L}$ and $I_r$ amplitude are similar ([6] and present results). The implications of these findings are quite interesting: a certain concentration of 5-HT able to stimulate the 5-HT$_4$ receptor, will cause a concomitant increase in both $I_{Ca,L}$ and $I_r$ in atrial cells. These two effects may cooperate in the arrhythmogenesis of the human atrium. In fact, as suggested by Kaumann [8], the increase in calcium current may lead to calcium overload, causing the transient inward current which underlies delayed afterdepolarizations (DADs) [35]. Epinephrine-induced DADs have been recorded in human atrial trabeculae [36]. DADs are a well known arrhythmogenic mechanism [37]; if their amplitude is sufficient they may reach the threshold giving rise to a spontaneous action potential (triggered activity). The increase in $I_r$ amplitude may contribute to this phenomenon. In fact, if $I_r$ is responsible for a diastolic depolarization, an increase in its amplitude can lead to a diastolic depolarization which, in its turn, may favor the attainment of threshold by a subthreshold DAD, thus causing an extrasystole, which may trigger the arrhythmia. In the presence of an arrhythmogenic substrate, such as a dilated atrium of an old patient [38], atrial fibrillation could develop.

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References


