Heterologously Expressed Serotonin 1A Receptors Couple to Muscarinic K+ Channels in Heart

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Heterologously expressed serotonin 1A receptors couple to muscarinic K^+ channels in heart

(vaccinia virus expression/guanine nucleotide-binding proteins/seven-helix receptors/atrial cells/acetylcholine)

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ABSTRACT **In cardiac atrial cells, muscarinic acetylcholine receptors activate a K^+ current directly via a guanine nucleotide-binding protein (G protein). Serotonin type 1A receptors may activate a similar pathway in hippocampal neurons. To develop a system in which receptor/G protein/K^+ channel coupling can be experimentally manipulated, we have used a highly efficient recombinant vaccinia virus vector system to express human serotonin 1A receptors in primary cultures of rat atrial myocytes. The expressed 1A receptors activated the inwardly rectifying K^+ conductance that is normally activated by the endogenous muscarinic acetylcholine receptors. Maximal responses to either agonist occluded further activation by the other agonist. The average activation time constants for serotonin were about 5 times slower than for acetylcholine. The data support suggestions that the intracellular signaling pathway from seven-helix receptors to G proteins and directly to ion channels is widespread in excitable cells. After a fraction of the G proteins are activated irreversibly by guanosine 5′-[γ-thio]triphosphate, subsequent transduction proceeds more efficiently. One possible interpretation is that multiple G-protein molecules are required to activate each channel. Vaccinia virus expression vectors are thus useful for expressing seven-helix receptors in primary cultures of postmitotic cells and have provided a heterologous expression system for the signaling pathway from seven-helix receptors to G proteins and directly to ion channels.**

After an appropriate agonist binds to any of the several seven-helix receptors, a guanine nucleotide-binding protein (G protein) is activated. In most known cases, the G protein then activates or inhibits an enzyme, and cell function changes within a few seconds. An alternative signaling pathway, which was first observed for muscarinic acetylcholine receptor (mAChR) stimulation of cardiac atrial cells, is direct activation of an ion channel by a G protein (1–5); in this pathway, cell function can be altered within 100–300 ms (6, 7). Indirect evidence suggests that several additional classes of receptors, G proteins, and ion channels participate in such a direct, relatively rapid receptor/G protein/ion channel pathway in a variety of excitable cells. Candidate receptors include serotonin (5-hydroxytryptamine, 5-HT) 1A, μ or δ opioid, adenosine A_1, γ-aminobutyrate $GABA_B$, dopamine D_2, somatostatin, and α_2-adrenergic receptors (8–11). Strong evidence for this suggestion would come from the demonstration that the several protein components of this pathway retain sufficient similarity to allow efficient and rapid signaling in a reconstituted system with receptors, G proteins, and ion channels from different cells of origin. Indeed, in inside-out patches, guanosine 5′-[γ-thio]triphosphate (GTP[γS])-stimulated $G_i\alpha$ subunits can directly activate (12) the inwardly rectifying K^+ channels normally activated by mAChRs, and probably adenosine A_1 and somatostatin receptors (13, 14), in atrium. We therefore sought to express an additional seven-helix receptor in atrial cells and tested for functional coupling to the endogenous K^+ conductance.

It has been reported that 5-HT type 1A receptors (5-HT_{1A}Rs) are coupled in various cells both to inhibition of forskolin-stimulated adenylate cyclase (15, 16) and to its stimulation (17). However, the latter conclusion has been criticized (16). In a heterologous system, stimulation of phospholipase C and inhibition of adenylate cyclase have been reported (18). We chose the 5-HT_{1A}R for heterologous expression because of the evidence that it can activate G_i and because it has been reported to activate K^+ channels in hippocampal neurons by a pertussis toxin-sensitive G protein and without soluble cytoplasmic intermediates (19).

The most appropriate host cell was the atrial myocyte, because (*i*) the direct receptor/G protein/K^+ channel pathway has been demonstrated most conclusively for this cell and (*ii*) although several techniques have been developed for stable expression of foreign proteins in clonal cell lines, inwardly rectifying G-protein-activated K^+ channels are not presently known in any cell lines. A previous study (20) showed that the vaccinia virus (VV) system allows for heterologous expression of K^+ channels in primary cultures, including atrial myocytes, at a density of 1 to 5 channels per μm^2. However, it was expected that higher expression levels would be required for seven-helix receptors in the pathway that involves direct coupling from G proteins to ion channels. In bullfrog atrial cells the average density of mAChRs (≈170 per μm^2 of cell membrane) exceeds that of K^+(ACh) channels by ≈200-fold (21); in AtT-20 lactotrophs the ratio of somatostatin S-14 receptors to K^+ channels is ≈130 (22). We have therefore adapted a highly efficient variant of the vaccinia system for expression of the 5-HT_{1A}R.

MATERIALS AND METHODS

Construction of a Recombinant Virus Encoding the 5-HT_{1A}R. For the construction of the recombinant plasmid pTM1-5HT1AR, the start codon of the 5-HT_{1A}R gene was inserted into an *Nco* I site of the VV insertional vector pTM1 (23) to correspond to the initiating ATG codon in the sequence of the encephalomyocarditis virus (EMCV). Therefore, a *Dde* I–*Bam*HI fragment (1.29 kilobases) within the coding region of the 5-HT_{1A}R gene was excised from pSVL-5HT1AR and ligated to a synthetic 5′ *Nco* I/3′ *Dde* I adaptor

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Abbreviations: 5-HT, serotonin (5-hydroxytryptamine); 5-HT_{1A}R, serotonin type 1A receptor; ACh, acetylcholine; mAChR, muscarinic ACh receptor; G protein, guanine nucleotide-binding protein; GTP[γS], guanosine 5′-[γ-thio]triphosphate; VV, vaccinia virus.

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that contained the sequence between the start codon and the *Dde* I site of the receptor coding region. pTM1 was digested with *Nco* I/*Bam*HI, dephosphorylated, and ligated to the adaptor/5-HT_{1A}R fragment. The recombinant plasmid pTM1-5HT1AR, amplified in *Escherichia coli* JM109 and purified by CsCl centrifugation, was inserted into the VV genome by homologous recombination *in vivo*. Thymidine kinase-negative L (Ltk^-) cells were infected with wild-type VV (WR strain from the American Type Culture Collection) at low multiplicity of infection (0.05 plaque-forming unit per cell) and subsequently transfected with pTM1-5HT1AR. After amplification in medium containing 5′-bromodeoxyuridine, successful recombinants were isolated by several rounds of plaque hybridization and grown to large scale (24).

Infection of Atrial Myocytes. Atrial myocytes were dissociated from the atria of rats at postnatal day 2 or 3 by using collagenase (25) and were incubated for 24–48 hr before infection. Cultures were simultaneously infected with the helper virus vTF7-3 and VV:5HT1AR (multiplicity of infection ≈ 10 each) for 30 min at room temperature, the virus inoculum was exchanged for medium, and the cells were incubated for 12–24 hr. Precautions were observed to prevent accidental exposure of personnel and to inactivate all virus before disposal.

Electrophysiological Recordings. Infected cells on coverslips were washed three times with Tyrode bath solution (135 mM NaCl/5.4 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/10 mM Hepes/5 mM glucose, pH 7.4). Patch pipettes for whole-cell recording (26) were pulled from Kimax borosilicate glass tubing and heat-polished to give input resistance values of 4–7 MΩ. The intracellular pipette solution was 140 mM KCl/2 mM $MgCl_2$/1 mM EGTA/5 mM Hepes/1 mM Na_2ATP/0.1 mM cAMP/200 μM GTP, pH 7.3. Currents were measured at room temperature (19–22°C) with a DAGAN 8900 (Dagan Instruments, Minneapolis) patch clamp and low-pass-filtered at 3 kHz. Stimulation, data acquisition, and analysis were performed using PCLAMP software (Axon Instruments, Foster City, CA) on an IBM PC. Agonist-induced currents were isolated by digitally subtracting capacitive transients and records in the absence of agonists. Drugs were applied from a fast flowpipe microperfusion system consisting of seven polyethylene tubes (each of 150-μm internal diameter). The parallel array of tubes was mounted on a micromanipulator and 100–150 μm from the cells. Change of solutions using this system was measured using K^+ concentration jumps in the presence of agonist and occurred in <50 ms.

Binding Assay. Ligand binding was performed (27) with lysed membranes in 50 mM Tris/1 mM $MnCl_2$, pH 8.2, for 60 min at room temperature. Nonspecific binding was determined in the presence of 10 μM 5-HT.

RESULTS

To obtain expression of the 5-HT_{1A}Rs at levels (21, 22) approaching those of the endogenous mAChRs (>100 per μm^2), we used a novel high-efficiency expression system based on infection with two recombinant VVs (Fig. 1) (23). One VV (vTF7-3) had been engineered to express the bacteriophage T7 RNA polymerase gene under the control of the early/late P7.5 vaccinia promoter (28). The second virus (VV:5HT1AR) carried (from 5′ to 3′) the T7 RNA polymerase promoter (Φ 10) and hairpin sequence, the 5′ untranslated region from encephalomyocarditis virus, the coding region of the human 5-HT_{1A}R gene (29), and the T7 terminator sequence (TΦ). In experiments to test the efficiency of expression, BSC-40 cells, COS-7 cells, and GH_3 cells were coinfected with vTF7-3 and VV:5HT1AR. Specific binding of tritiated 5-HT and 8-hydroxy-2-dipropylaminotetralin revealed ≈3 pmol/mg of membrane protein, corresponding to

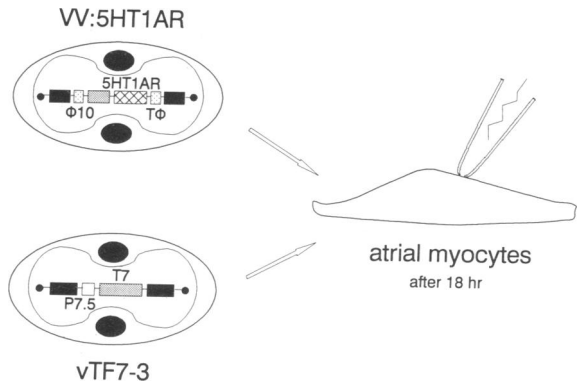

FIG. 1. Coinfection procedure for high-level expression of 5-HT_{1A}Rs in atrial cells using two recombinant VVs. Φ10 and TΦ, phage T7 RNA polymerase promoter and terminator sequences; P7.5, P7.5 vaccinia promoter. See *Materials and Methods* for further explanation.

≈1.5×10^5 5-HT_{1A}Rs per COS-7 cell (or 85 per μm^2 of cell membrane, if most of the receptors are on the plasma membrane).

When noninfected control cells were voltage-clamped with the external K^+ concentration ($[K^+]_o$) raised to 25 mM, application of ACh (5 μM) from a fast microperfusion system induced prominent inward currents with amplitudes of −655 ± 92 pA (mean ± SEM; n = 23) at −90 mV. Application of 5-HT (5 μM) in these control cells never elicited any detectable responses (n = 25; Fig. 2*A*). In contrast, 12–18 hr after atrial myocytes were coinfected with the helper virus vTF7-3 and VV:5HT1AR, they responded with an inward current upon application of both ACh (−628 ± 91 pA; n = 17) and 5-HT (−288 ± 30 pA; n = 17; Fig. 2*B*). Some of the infected atrial cells were still beating but were identified by distinct morphological changes, such as rounding up and cytoplasmic inclusions. Approximately 30% of the 224 obviously infected cells that were studied responded to 5-HT. Voltage ramp recordings in the presence of both ACh and 5-HT (Fig. 2 *C* and *D*) showed strong rectification for inward currents, negative to the equilibrium potential for K^+ (E_K = −43 mV). The inwardly rectifying current induced by both agonists was highly selective for K^+, since the reversal potential decreased with increasing $[K^+]_o$ and was close to E_K, as predicted by the Nernst equation. In a different voltage protocol the membrane potential of infected atrial cells was stepped to hyperpolarizing levels in the presence of the agonists and the current responses were plotted against the step potentials (Fig. 2*E*). Again the current/voltage (I/V) relations for the action of ACh and of 5-HT in the same cell were identical and inwardly rectifying. In addition, the voltage jump relaxations for the two agonists exhibited the same time constants (τ = 19 ms at V_c = −120 mV), similar to the value reported previously for $I_{K[ACh]}$ in frog and guinea pig atrial cells (30, 31). Current with this characteristic I/V relationship in coinfected cells could be mimicked by the selective 5-HT_{1A}R agonist 8-hydroxy-2-dipropylaminotetralin. These results indicate that in coinfected atrial cells, newly expressed 5-HT_{1A}Rs mediate the activation of a K^+ conductance identical or very similar to the endogenous current $I_{K[ACh]}$, which is activated by ACh.

In an additional series of experiments using 5- or 10-s pulses of agonists between 0.05 and 50 μM, we studied occlusion of the responses. Concentration–response relations revealed half-maximal concentrations of 0.36 μM for ACh and 0.11 μM for 5-HT and desensitization in responses to concentrations above 25 μM (Fig. 3*A*). A maximal response evoked by either one of the two agonists (5 μM) occluded additional increases during application of the other

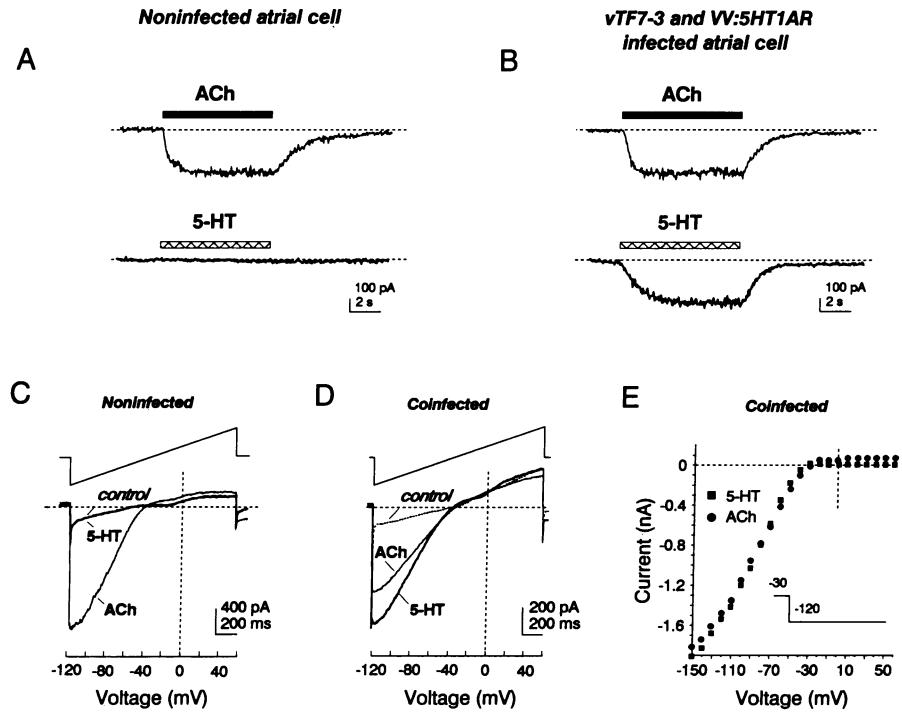

FIG. 2. After coinfection with vTF7-3 and VV:5HT1AR, atrial myocytes respond to 5-HT with a current that corresponds to $I_{K[ACh]}$. Cells were voltage-clamped at −90 mV (*A* and *B*), or stimulated with a voltage ramp between −120 and +60 mV (*C* and *D*) and $[K^+]_o$ was raised to 25 mM. Horizontal bars indicate the time of drug application. (*A* and *C*) Noninfected cells and cells infected with wild-type VV (data not shown) respond to ACh (10 μM) but not to 5-HT (10 μM) with an inwardly rectifying current. (*B* and *D*) Coinfected cells after 14 hr respond to both agonists with current primarily in the inward direction. (*E*) Superimposed current/voltage relationship for whole-cell currents induced by ACh and 5-HT, measured at the end of 200-ms hyperpolarizing voltage steps from −30 mV to −120 mV.

agonist (Fig. 3*B*), which suggests that the two agonists compete for the same component in the receptor/G protein/K^+ channel pathway. Thus, both the endogenous mAChRs and the VV-expressed 5-HT_{1A}Rs converge onto the same population of K^+ channels.

The activation of $I_{K[ACh]}$ appears to be mediated directly by a G protein, probably a $G_i\alpha$ subunit (12, 32), independently of any difussible cytoplasmic messengers. Strong evidence for direct coupling was provided by (*i*) measurements on single channels in excised cell-free membrane patches from atrial muscle (4, 5) and (*ii*) the rapid time course of activation and deactivation. The current evoked by ACh (5 μM) in our preparation activated with a time constant of τ_{act} = 317 ± 12 ms (n = 32), in agreement with previous data (6, 7). The deactivation time constant was τ_{deact} = 965 ± 75 ms (n = 23; Fig. 4*B*). Interestingly, current responses mediated by the expressed 5-HT_{1A}Rs in coinfected atrial cells (5 μM 5-HT) showed similar deactivation rates upon removal of the agonist, with τ_{deact} = 924 ± 39 ms (n = 16), but in addition to the typically smaller current amplitudes, more variable and usually slower activation time constants, τ_{act} = 1682 ± 180 ms (n = 12; Fig. 4*B*), were observed.

The different activation rates for ACh and 5-HT responses could arise from the probable lower density of 5-HT receptors or from another mechanism, such as a less effective receptor/G protein association. To investigate this difference further, we uncoupled the receptors from the rest of the pathway by internally perfusing the nonhydrolyzable GTP

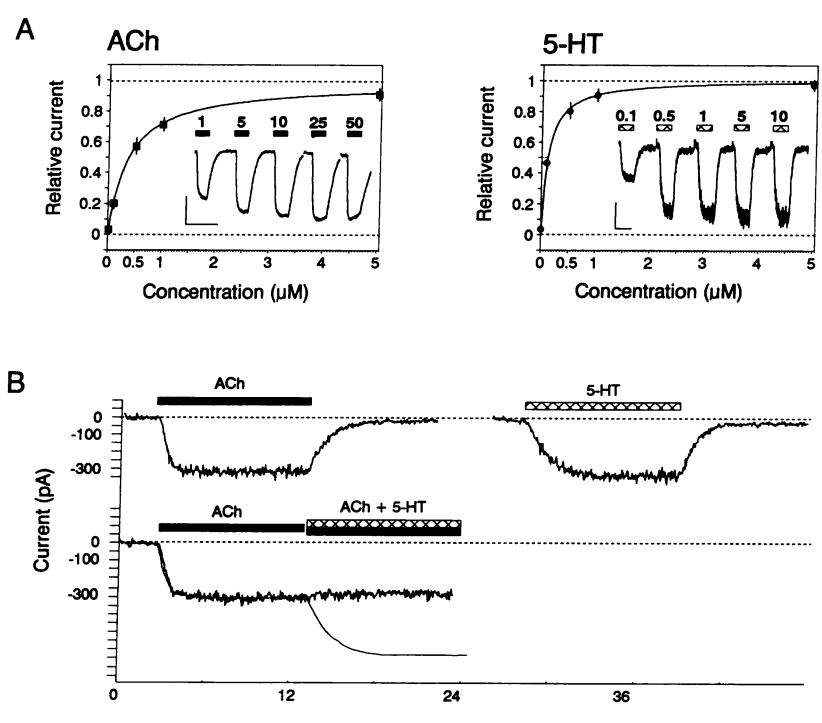

FIG. 3. Maximal responses to 5-HT and ACh occlude each other. (*A*) Concentration–response relationships for both ACh and 5-HT in two different coinfected atrial cells. Data represent the mean ± SEM of normalized current amplitudes (I_{max} = response to 25 μM agonist) of four cells each. The raw traces are consecutive current responses, at a holding potential of −90 mV, to 5- or 10-s pulses of each agonist (in μM). Scale bars: 500 pA (ACh), 100 pA (5-HT), 10 s. The curves are the best fit of the data to $I = I_{max} \times [agonist]/(EC_{50} + [agonist])$ with EC_{50} = 360 nM (ACh) or 110 nM (5-HT). (*B*) When a maximal current response is induced by ACh (5 μM), additional application of 5-HT (5 μM) in a coinfected atrial cell produces no further response, and vice versa. Superimposed line represents the expected response if the two responses were independent of each other and additive.

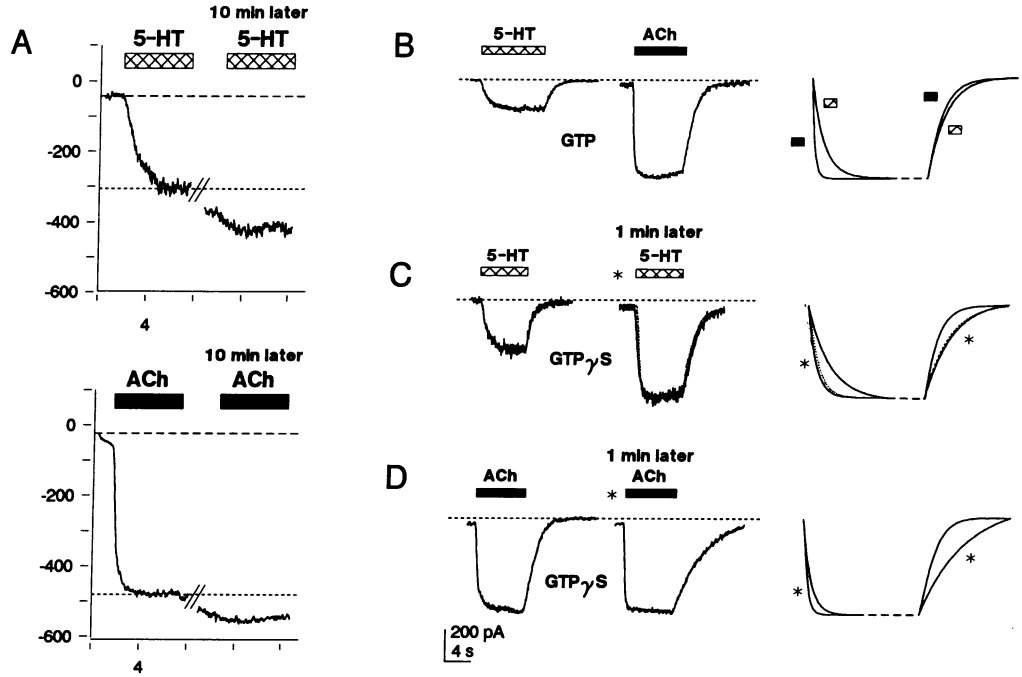

FIG. 4. Intracellular perfusion of GTP[γS] indicates the involvement of a G protein for the actions of both ACh and 5-HT (5 μM) in coinfected atrial cells. (*A*) Persistent activation of $I_{K[ACh]}$ by GTP[γS]. Neither agonist produces a significant response after complete activation of $I_{K[ACh]}$ with 500/50 μM internal GTP[γS]/GTP. (*B–D*) Kinetic analysis of the activation and inactivation rates of the currents induced by both agonists. The traces in *B* represent typical responses to 5-HT and ACh in a cell perfused with 200 μM GTP. Activation time constants (τ_{act}) are 1250 ms (5-HT) and 300 ms (ACh); inactivation time constants (τ_{inact}) are 1100 ms (5-HT) and 1020 ms (ACh). Intracellular perfusion with GTP[γS]/GTP (1/0.2 mM) changes the onset and offset rates of $I_{K[ACh]}$ for both agonists, even before persistent current activation develops fully. In *C*, for 5-HT-induced currents: τ_{act} = 1490 ms (0 min), 550 ms (1 min); τ_{inact} = 920 ms (0 min), 1950 ms (1 min). Response to ACh recorded 20 s earlier is superimposed (dotted line). In *D*, for ACh-induced currents: τ_{act} = 340 ms (0 min), 195 ms (1 min); τ_{inact} = 1500 ms (0 min), 4700 ms (1 min). Exponential fits on the right are normalized in amplitude; the traces marked with an asterisk represent recordings taken at later times.

analog GTP[γS] through the patch pipette. The experiments revealed two different time-dependent processes. First, as expected, a persistent activation of $I_{K[ACh]}$ by GTP[γS] developed slowly over several minutes, even in the absence of the agonists (33). After this persistent activation by GTP[γS] reached a plateau, addition of ACh or (in coinfected cells) 5-HT produced only a small additional current (Fig. 4*A*), demonstrating that an activated G protein can open the same channel. The time required for complete activation of receptor-independent $I_{K[ACh]}$ varied from 4 to 20 min depending on the GTP[γS]/GTP ratio (2:1 to 10:1) in the pipette solution and on whether agonists had been applied intermittently (33).

A second, unexpected set of phenomena occurred at early stages during the perfusion with GTP[γS]. There was (*i*) an increase in the rate of current activation and (*ii*) a decrease in deactivation rate in response to either agonist (Fig. 4 *B–D*) and (*iii*) especially for 5-HT, an increase in current amplitude. These effects were never observed when GTP[γS] was absent from the internal solution. GTP[γS] perfusion dramatically increased the typically slow and small responses mediated by the heterologously expressed 5-HT_{1A}Rs. In a minority of cells, extremely slow 5-HT-induced responses (τ_{act} > 3000 ms) developed amplitudes and kinetics similar to the currents elicited by the same concentration of ACh (τ_{act} < 500 ms). We emphasize that the measurements on heterologously expressed 5-HT_{1A}Rs readily revealed a kinetic effect that is also present for the endogenous muscarinic response. With both ACh and 5-HT, the faster activation corresponds to an increase of 1–2 s^{-1} in the rate constant for activation.

DISCUSSION

The present data, showing that human 5-HT_{1A}Rs couple to the muscarinic pathway in rat atrial myocytes, confirm the suggestion that 5-HT_{1A}Rs couple to K^+ channels via a direct G protein–channel interaction. The occlusion data show that the newly expressed 5-HT_{1A}Rs activate the same K^+ channels as the endogenous muscarinic AChRs, thus suggesting that they activate very similar G proteins.

Because the experiments employed the high temporal resolution of electrical recordings, they also showed (*i*) that, under the conditions used, activation by 5-HT proceeds more slowly than activation by ACh and (*ii*) that deactivation proceeds at about the same rate. These kinetic phenomena were studied further, and an additional effect was noted. After a fraction of the G proteins are activated irreversibly by GTP[γS], subsequent transduction proceeds more efficiently. This effect was present for both the endogenous ACh responses and for the newly expressed 5-HT responses. In present schemes for activation of this pathway, an activated receptor promotes nucleotide exchange at the heterotrimeric G_k protein and subsequent dissociation into GTP-activated α[GTP] and βγ subunits. The finding that irreversible G-protein activation by GTP[γS] enhances the efficiency of transduction by a subsequent pulse of agonist could be interpreted in a straightforward fashion if the channel required interaction with several (*n*) α[GTP] subunits rather than with a single one. We cannot definitely state the identity of the rate-limiting step for activation of the channel; it could be: (*i*) GDP/GTP exchange, (*ii*) α[GTP]–βγ dissociation, or (*iii*) diffusion of the activated α subunit toward the channel. What is important is that this rate-limiting step is eliminated for a Gα subunit that has GTP[γS] bound to it. As time passes in the presence of GTP[γS], more and more of the *n* Gα subunits required to activate a single channel become activated. With subsequent agonist applications, fewer Gα subunits need to undergo the rate-limiting step and activation becomes more rapid. Eventually, when all *n* Gα subunits for a given channel are irreversibly activated, the channel becomes permanently activated.

In this model, the channel closes upon GTP hydrolysis at any of the n independent subunits associated with it. Thus the rate of channel closing decreases as the Gα subunits become permanently bound with GTP[γS]. When only 1 of the n Gα subunits retains GTPase activity, the channel deactivation rate decreases by n-fold from the value with all subunits able to hydrolyze GTP. For the data in Fig. 4, n is in the range 2–4; for all of our averaged data, $n = 2.9$. This model invites speculation that the channel, like many voltage-dependent K^+ channels, has n independent subunits, each capable of interacting with a Gα subunit. The model predicts a nonlinear dependence of $I_{K[ACh]}$ on guanine nucleotide concentration. Such an effect might have escaped notice in the past because of technical difficulties in accurately controlling the rather low GTP concentrations ($K_m \approx 10^{-7}$ M) involved. Recent studies do suggest that the dependence of activation on guanine nucleotide concentration is characterized by a Hill coefficient of 2–3 (33, 34). A detailed scheme involving these ideas must also account for the Hill coefficient near 1 usually observed for activation of $I_{K[ACh]}$ (see also Fig. 3*A*). Other mechanisms for the effects of hydrolysis-resistant GTP analogs—e.g., involving GTPase-activating proteins (35), arachidonate metabolites (36, 37), and $\beta\gamma$ subunits—are possible.

It was previously suggested that activation of K^+ channels by muscarinic receptors involves several independent but identical rate-limiting steps (6, 38). Our experiments suggest that these steps might involve multiple G-protein molecules interacting with a single channel.

We may ask whether ACh acting on atrial mAChRs and 5-HT on the heterologous 5-HT_{1A}Rs activate the same set of G proteins. Yatani *et al.* (12) have shown that in inside-out atrial patches, $G_i\alpha1$, $G_i\alpha2$, and $G_i\alpha3$, when activated by [GTPγS], all efficiently stimulate the same K^+ channels. Activated $G_s\alpha$ was not effective, and $G_o\alpha$, as purified, stimulated about 5% as effectively as did any $G_i\alpha$ (4). There is compelling evidence that 5-HT_{1A}Rs do activate G_i (i.e., inhibit forskolin-stimulated adenylate cyclase activity) in a number of cell types (15, 16, 18); in a heterologous expression system, they are also coupled to phospholipase C, albeit with an ≈50-fold higher EC_{50}. Coupling to phospholipase C may be mediated by G_o (39). We conclude that the principal pathway for 5-HT_{1A}R stimulation of the K^+ current is via direct ligand activation by one or several atrial $G_i\alpha$ isoforms, all of which are expressed in human atria (40).

VV vectors presently provide the most appropriate heterologous expression system for postmitotic vertebrate cells in primary culture. Because VV directs expression in a high percentage of the cells in a given culture, this system is well suited for expression for single-cell studies. VV systems may prove useful for reconstitution of the entire functional seven-helix/G protein/ion channel pathway.

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