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Research report

Functional differences between splice variants of the murine 5-HT_{3A} receptor: possible role for phosphorylation

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Abstract

The murine 5-HT_{3A} receptor subunit is expressed as either of two splice variants which are differentially regulated in vivo. The difference resides in a six-amino acid sequence within the cytoplasmic loop between transmembrane regions 3 and 4, which is present in the long form but not the short form. No physiological roles have yet been ascribed to the two splice variants. Whole cell patch clamp recording from transfected HEK 293 cells stably expressing either long or short form receptors showed very similar responses under control conditions. However, inclusion of 1 mM cAMP (activator of protein kinase A) in the patch pipette caused an initial increase in the desensitization rate of the long form, but a decrease in the short form. With the addition of 100 nM phorbol 12-myristate 13-acetate (PMA; activator of protein kinase C) to the pipette solution, responses elicited with 1 μM 5-HT revealed an increase in the current amplitude in the long but not the short form of the receptor. Over a longer time period, inclusion of PMA in the patch-pipette caused a faster run down of peak current amplitude in response to 30 μM 5-HT in the long form but did not affect the short form; there was no observed long-term effects of cAMP. We conclude that the long and short forms of the 5-HT_3 receptor are differentially modulated by agents that activate PKA and PKC. These different patterns of modulation could have markedly divergent consequences on receptor function.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Serotonin receptors

Keywords: 5-HT_3 receptor; Splice variant; Phosphorylation; Desensitization; Whole-cell patch-clamp; Down-regulation

1. Introduction

The 5-HT_3 receptor is a member of the cys-loop family of ligand-gated ion channels [14]. In common with other members of this group, it consists of five subunits surrounding a central pore [2], each subunit comprising of a N-terminal (extracellular) ligand-binding domain, four putative membrane-spanning domains (M1–M4), of which M2 is thought to line the ion channel, and a large cytoplasmic loop between M3 and M4 [17]. The 5-HT_3 receptor was for some years believed to form functional homopentamers in vivo as only one subunit as originally identified [14]. Recently, however, a second subunit, the 5-HT_{3B} receptor subunit, has been cloned [3], and characterization of heteromeric receptors can account for some previously unexplained properties of neuronal 5-HT_3 receptors. However, there is still evidence that in some systems at least (e.g., mouse neuroblastoma N1E-115 cells and DRG neurones) 5-HT_3 receptors can exist as homo-oligomers of 5-HT_{3A} receptor subunits. It is of interest, therefore, that two splice variants of the 5-HT_{3A} receptor, differing by only six amino acids, have been isolated from

Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol-bis (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; epsp, excitatory post synaptic potential; 5-HT, 5-hydroxytryptamine; 5-$HT_{3A(a)}$, 5-hydroxytryptamine$_3$ receptor subunit A (long 6 form); 5-$HT_{3A(b)}$, 5-hydroxytryptamine$_3$ receptor subunit A (short form); I_{max}, peak current amplitude of 5-HT-induced current; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G

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the mouse [12,22]. These are differentially expressed during development [15] and can be differentially regulated in vitro [5]. Differences in the efficacy of the 5-HT_3 receptor agonists, 2-methyl 5-HT and *m*-chlorophenylbiguanide, have been observed for recombinant, homomeric long or short forms of the 5-HT_{3A} receptor [4,16,18], but the characteristics of this receptor when activated by the endogenous agonist 5-HT appear identical, and no functional differences have yet been ascribed to the two splice variants in vivo or in vitro [8]. The current study was designed to investigate differences between the splice variants and in particular to explore whether intracellular application of cAMP (an activator of protein kinase A; PKA) or phorbol 12-myristate 13-acetate (PMA, an activator of protein kinase C, PKC) would result in different functional characteristics following activation of the receptor.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney cells (HEK 293) were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK) and maintained in DMEM/Dulbecco's F-12 medium supplemented with 10% foetal calf serum (Sigma, Poole, UK) at 37°C in 7% CO_2 100% relative humidity and passaged every 4–6 days, when confluent. HEK 293 cells were stably transfected with DNA coding for 5-HT_3R-$A_{(a)}$ (long form) or 5-HT_3R-$A_{(b)}$ (short form) in the eukaryotic expression vector pRc/CMV (InVitrogen, Abingdon, UK) as previously described [11]. For electrophysiological recording, cells were plated at low density onto 35-mm culture dishes (Falcon, Becton-Dickinson UK, Oxford, UK) and used in experiments within 18–32 h.

2.2. Electrophysiological recording

5-HT-induced currents were recorded from isolated single cells in the whole-cell attached configuration as previously described [8]. Briefly, cells were patched with borosilicate glass micropipettes (Clark Electromedical Instruments, Reading, UK), back filled with intracellular solution containing 140 mM CsCl, 1.0 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1.1 mM EGTA (10 nM free Ca^{2+}), 10 mM Hepes, pH 7.2, at 24°C in the presence or absence of 1.0 mM cAMP (adenosine 3′,5′-cyclic monophosphate, sodium salt; an activator of PKA), 100 nM PMA (phorbol 12-myristate 13-acetate; and activator of PKC), 1 mM cGMP (guanosine 3′,5′-cyclic monophosphate, sodium salt; an activator of PKG and analogue of cAMP) or 100 nM 4-α-phorbol (4-α-phorbol-12,13-didecanoate, an inactive analogue of PMA). These compounds were used to examine specificity in preference to protein kinase inhibitors some of which appear to act directly on the receptor; the broad range protein kinase inhibitor H7, for example, has no effect on currents mediated by 5-HT_3 receptors when applied intracellularly, but does block them when applied extracellularly [6], whilst the inhibitory effects of PKC inhibitor bisindoylmaleimide are not apparent when using the equipotent but less specific inhibitor, staurosporine [8].

Micropipettes had resistances of 2–4 MΩ. Currents were recorded at 20–24°C using an EPC-9 amplifier (HEKA Electronik, Darmstadt, Germany) controlled by Pulse software (HEKA, version 7.85). Cells were clamped at −60 mV and continuously perfused (3–5 ml/min) with extracellular solution containing 140 mM NaCl, 5.4 mM KCl, 2.0 mM $MgCl_2$, 1.8 mM $CaCl_2$, 30 mM D-glucose, 10 mM Hepes, pH 7.2. 5-HT (30 μM unless otherwise stated) was applied via a 'U'-tube device [20] or DAD-12 perfusion system [10], allowing a complete change of agonist concentration at the cell membrane within 100 ms [19]. The first application of 5-HT was given within 10 s of the cell membrane being ruptured. Applications were then given at 4-min intervals to allow washout of 5-HT and complete resensitization of the receptors prior to the next application. Liquid junction potentials arising at the tip of the patch pipette were calculated by the method of Barry and Lynch [1], and potential measurements corrected post hoc.

2.3. Analysis of data

Agonist concentration–response data were fitted with a logistic function using Kaleidagraph (Abelbeck Software):

$$I_n = I_{max} \cdot [5\text{-HT}]^{n_H} / (EC_{50}{}^{n_H} + [5\text{-HT}]^{n_H})$$

where I_n = normalised response, I_{max} is maximal response, EC_{50} is concentration of agonist required for half maximal effect and n_H is the Hill coefficient.

Current–voltage relationships were fit with second order polynomials using Kaleidagraph (Abelbeck Software) and reversal potentials determined from the point at which these curves bissected the abscissa.

The half time of desensitization, $t_{1/2}$, represents the time taken for the current to decay to half of its peak value in the continued presence of 5-HT.

All data are quoted as mean±S.E.M. for *n* independent experiments. Statistical analyses were performed on normalised data by Student's *t*-test or ANOVA followed by Sheffé's test for multiple comparisons using StatView (Abacus Concepts, Berkleley, CA, USA), as appropriate. A *P* value of <0.05 was taken to be statistically significant.

2.4. Drugs and reagents

All cell culture reagents were obtained from Gibco-BRL (Paisley, UK), except fetal calf serum which was from Sigma (Poole, UK). 5-HT hydrochloride was from Re-

search Biochemicals (St. Albans, UK). All other reagents were obtained from Sigma.

3. Results

Application of a supramaximal concentration (30 μM) of 5-HT, to cells voltage clamped at −60 mV, evoked rapid inward currents in all cells tested (20–80% rise time for current activation was 83±9 ms, $n=9$, and 74±3 ms, $n=8$, for long and short splice variant receptors, respectively). Dose–response relationships revealed similar EC_{50} values (2.6±0.3 μM, $n=10$, and 2.6±0.2 μM, $n=9$), current–voltage relationships (data not shown) and reversal potentials (−2.7±0.5 mV, $n=9$, and −2.9±0.6 mV, $n=8$) for long and short splice variant receptors, respectively.

Time-dependent changes were observed for both peak current amplitude (I_{max}) and the half-time of desensitization ($t_{1/2}$) in response to repeated 30 μM 5-HT applications (Fig. 1). In both long and short splice variants, there were initial increases in $t_{1/2}$, which reached a maximum 4 min after the cell membrane was ruptured, and thereafter decayed with an exponential time course (Fig. 1B). I_{max} did not change over the first 4 min, but thereafter decayed in a near-linear fashion (Fig. 1A). Example traces are shown in the inset to Fig. 1B.

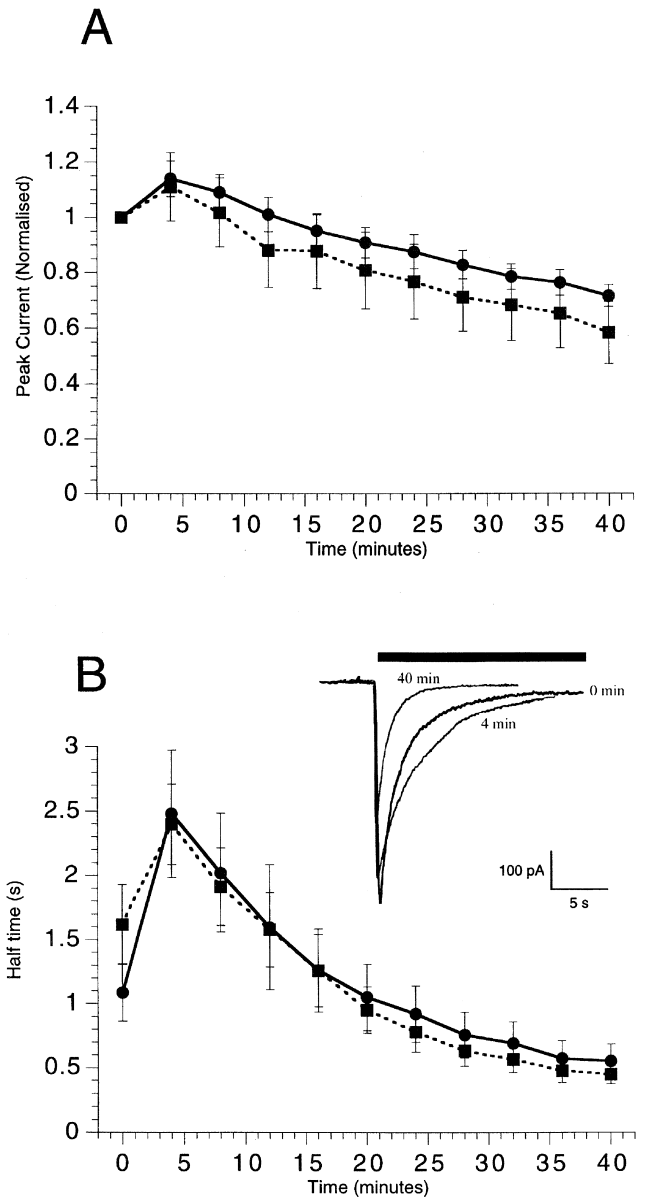

Fig. 1. Time course of I_{max} (A) and $t_{1/2}$ (B) of whole-cell currents recorded by patch-clamp from long ($n=9$) and short ($n=8$) 5-HT_{3A} receptors stably expressed in HEK 293 cells. Data in (A) have been normalised to the first response ($t=0$). At no time point were any significant differences observed between the long and short forms of the receptor (Student's *t*-test). Representative example traces are shown in the inset.

3.1. Effect of cAMP and PMA

The presence of PMA or cAMP in the intracellular solution had no effect on the EC_{50}, current–voltage relationship, reversal potential or rise time of the long or short splice variants (data not shown). However these compounds did have differential effects on the time-dependent changes in I_{max} and $t_{1/2}$. As these parameters change with different time courses (see above) they were treated as separate phenomena.

In both splice variants, I_{max} was similar to control values over the first 4 min of the experiment when in the presence of cAMP or PMA; however, over the remaining 40 min of the experiments, inclusion of 100 nM PMA in the patch pipette increased the rate of decay of I_{max} ('run-down') in the long form (Fig. 3A) but not the short form (Fig. 3B) of the receptor when using a maximal concentration of 5-HT (30 μM). This effect was manifest after 8 min and continued throughout the duration of the experiment. The effects of PMA when using responses elicited with 3 μM 5-HT (approx. EC_{50} concentration) were not significantly different to these, but at a sub-EC_{50} concentration of 5-HT (1 μM) PMA caused an increase in current in the long but not the short form of the receptor (Fig. 4). No difference to control was seen in I_{max} when 1 mM cAMP was included in the patch pipette.

The initial responses to 5-HT in the two splice variants were indistinguishable under control conditions but were significantly different in the presence of cAMP, as inclusion of 1 mM cAMP in the pipette solution decreased the initial rise of $t_{1/2}$ in the long form when compared to control conditions, whereas there was no significant difference in $t_{1/2}$ in the short splice variant of the receptor (Fig. 2A). In contrast, inclusion of 100 nM PMA in the pipette solution had no effect on the initial response in either splice variant as compared to control conditions. Following the initial changes in $t_{1/2}$, no difference was observed in this parameter for either splice variant in the presence of PMA or cAMP over the remaining 40 min of the experiment (Fig. 3C, D).

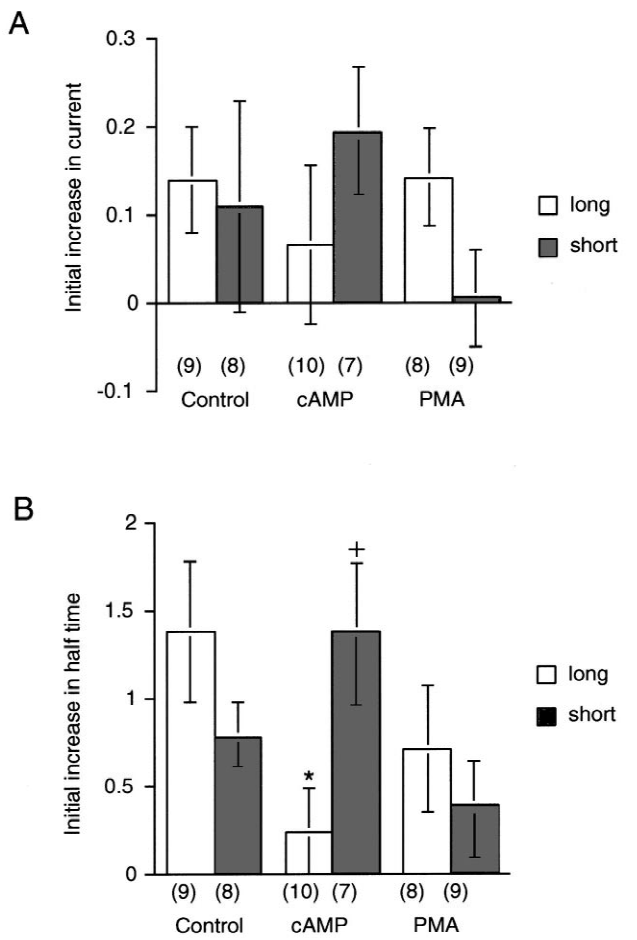

Fig. 2. Histograms showing the magnitude of the initial increase in I_{max} (A) and $t_{1/2}$ (B) of whole-cell currents of long (open bars) and short (filled bars) forms of the 5-HT_{3A} receptor under control conditions and in the presence of 1 mM cAMP (cAMP) or 100 nM PMA (PMA) in the patch pipette. Figures in parentheses refer to the number of observations. *Significantly different to control; $^{+}$significantly different to long form.

3.2. Effect of cGMP and 4-α-phorbol

To examine the specificity of the effects of cAMP and PMA we also examined the effect of adding cGMP and 4-α-phorbol to the intracellular solution. cGMP is an analogue of cAMP that naturally activates PKG but not PKA, and 4-α-phorbol is an inactive analogue of PMA. Changes in I_{max} and $t_{1/2}$ in response to 5-HT application were not significantly different to control when either of these compounds were present in the intracellular solution (Fig. 5).

4. Discussion

The present study demonstrates that many of the functional characteristics of homomeric 5-$HT_{3A(a)}$ (long) or 5-$HT_{3A(b)}$ (short) receptors activated by the endogenous agonist 5-HT do not differ: EC_{50} values, current–voltage relationships, reversal potential and rise times are indistinguishable, as previously reported by Glitsch et al. [8]. However, examination of the changes in the rate of recovery from desensitization and peak current with time ('run down'), has revealed that these properties are differentially effected by agents that modify phosphorylation, suggesting that receptors containing the different splice variants could have distinct physiological roles in vivo.

The changes in peak current amplitude and desensitization rate over time (Fig. 1) suggest that these parameters may be regulated in vitro by different intracellular modulatory mechanisms. It is possible that low molecular weight intracellular regulator(s) diffuse from the cell during the first 4 min of recording, thus causing the observed initial decrease in the rate of desensitization (increase in $t_{1/2}$), whilst higher molecular weight regulator(s) take much longer to diffuse out of the cell, resulting in the long time course of decay in peak current and $t_{1/2}$. There is no apparent relationship between changes in peak current and changes in half time, and these parameters have differing time courses of decay (linear versus exponential), indicating that these are distinct phenomena. A similar initial decrease in the rate of desensitization (i.e., increase in half time) and increase in peak current have also been noted by Yakel et al. [23] when recording 5-HT_3 receptor-mediated currents in NG108-15 cells. However, these authors noted that the rate of desensitization tended to stabilise after the first 10 min, whereas in the current study both parameters continued to decline with time (although with differing time-courses) to the end of the experiment (40 min). This discrepancy may simply be due to differing levels of the putative high-MW intracellular modulators in the different cell lines.

4.1. Effect of cAMP

Inclusion of 1 mM cAMP in the patch pipette had a markedly different effect on the initial increase in half time in the long splice variant compared to the short splice variant: in the long form this initial increase was severely unchanged (i.e., the rate of desensitization was increased), whereas in the short form the rate of desensitization was decreased. The former data agree well with those of Yakel and Jackson [24] and Yakel et al. [23] who found that activators of cAMP increase the rate of desensitization in hippocampus and in NG108-15 cells. These latter cells express both long and short splice variants of the 5-HT_{3A} receptor, and the proportion of each is known to vary [5]; thus, to explain their data in conjunction with ours, we would propose that the long splice variant of the receptor was dominant in these experiments.

The major effect of cAMP in animal cells is to activate PKA, which can then phosphorylate selected proteins. The intracellular loop of the 5-HT_{3A} receptor subunit possesses a concensus sequence for PKA which is located about 35 amino acids to the C-terminal side of the splice site, i.e.,

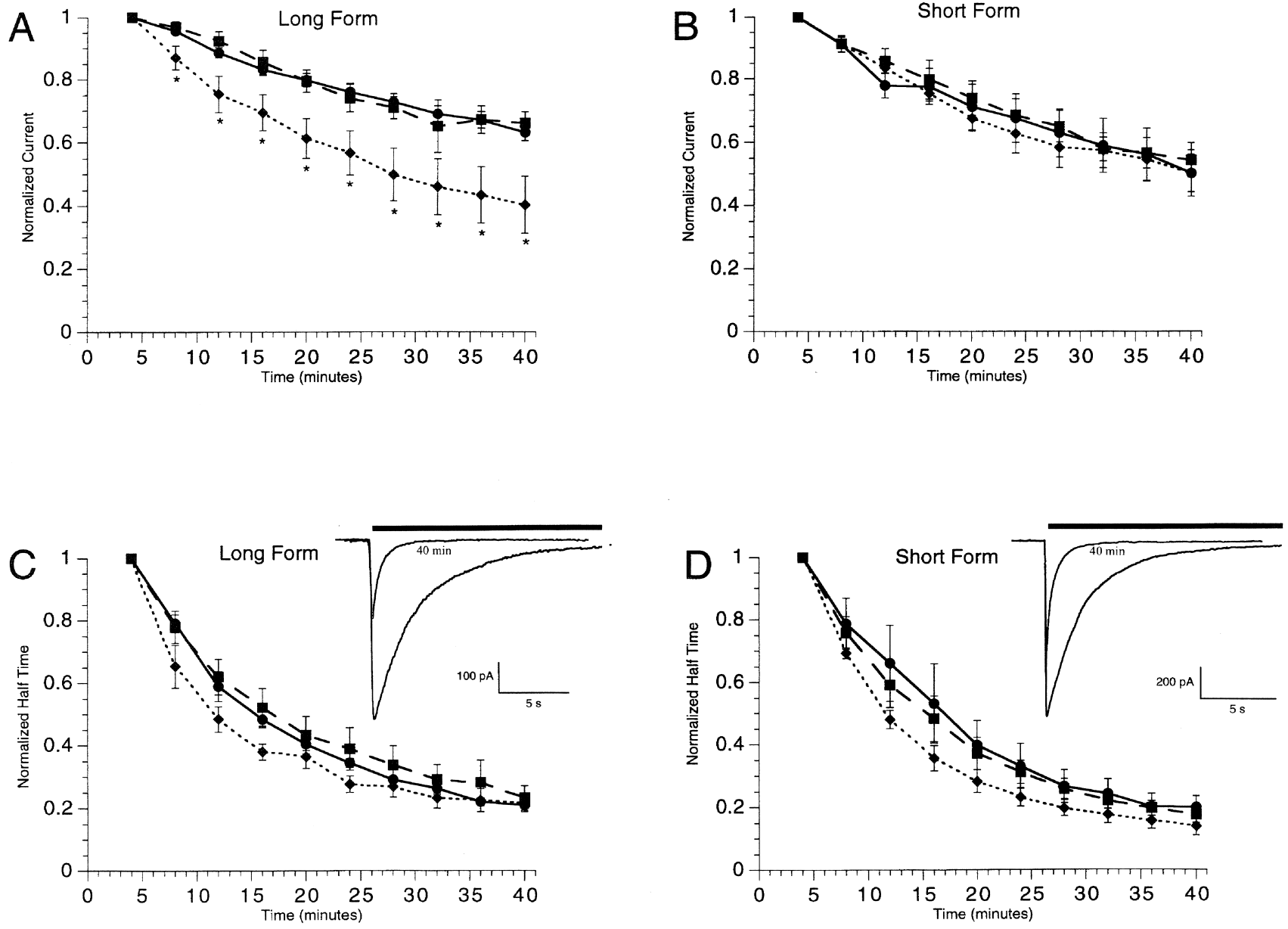

Fig. 3. Decay of I_{max} in long form (A) and short form (B), and of $t_{1/2}$ of long (C) and short (D) form of 5-HT_{3A} receptor whole-cell currents under control conditions (long, $n=9$; short, $n=8$) and in the presence of 1 mM cAMP (long, $n=10$; short, $n=7$) or 100 nM PMA (long, $n=8$; short, $n=9$). Data are normalised to the second response ($t=4$ min). *Significantly different to control. Example traces at 4 and 40 min in the presence of 100 mM PMA for the long (LHS) and short (RHS) forms of the receptor are shown in insets.

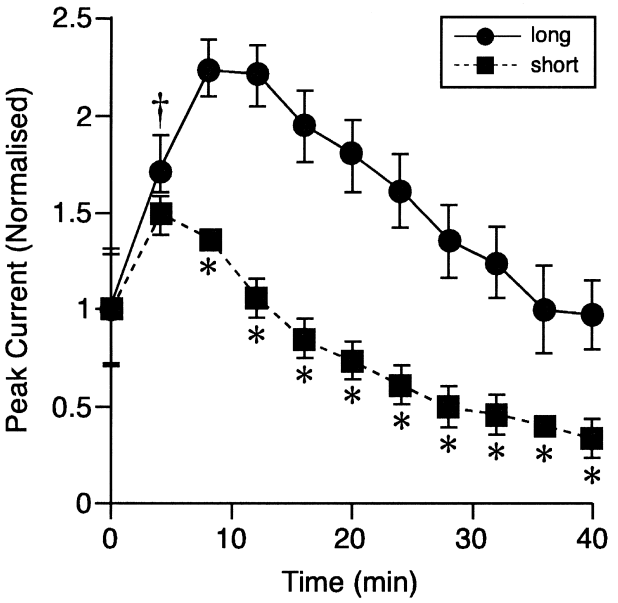

Fig. 4. Time course of $I_{5\text{-HT}}$ of whole-cell currents recorded by patch-clamp from long form (●, $n=9$) and short form (■, $n=8$) of murine 5-HT_{3A} receptors stably expressed in HEK 293 cells to repeated exposure to applications of 1 μM 5-HT in the presence of 100 nM PMA. Data have been normalised to the first response ($t=0$). *Significantly different to long form.

on both the splice variants. Is it therefore possible that PKA phosphorylates this sequence, yet results in opposite effects on the rate of desensitization of 'long' and 'short' receptors? The structures of this protein is not yet known but the extra amino acids present in the long but not the short form of the receptor could certainly have significant effects on the structure of the intracellular loops, and therefore significantly change the availability to enzymes of potential phosphorylation sites. Alternatively, PKA could phosphorylate one or more auxillary proteins, which then differentially modulate the two splice variants; some evidence which would support this hypothesis is the discovery of proteins associating with the 5-HT_3 receptor when it is purified from rat brain [7]. It is also possible that a phosphorylated receptor or auxillary protein could affect the local [Ca^{2+}]; changing intracellular [Ca^{2+}] has significant effects on the rate of 5-HT_3 receptor desensitization [13]. Further experiments are required to test these hypothesise but it is important to note that an increase in the rate of desensitization, as observed in the 'long' form, would be inhibitory in vivo, as this would reduce the likelihood of spatial or temporal summation of epsps in the post-synaptic cell, and therefore decrease the probability of it firing in response to a given pre-synaptic event. In contrast, a decrease in the rate of desensitization, as observed in the short form, would have the opposite effect. Thus activation of 5-HT_3 receptors combined with stimulation of the adenylate cyclase pathway would have markedly different functional consequences depending on which splice variant the cell is expressing.

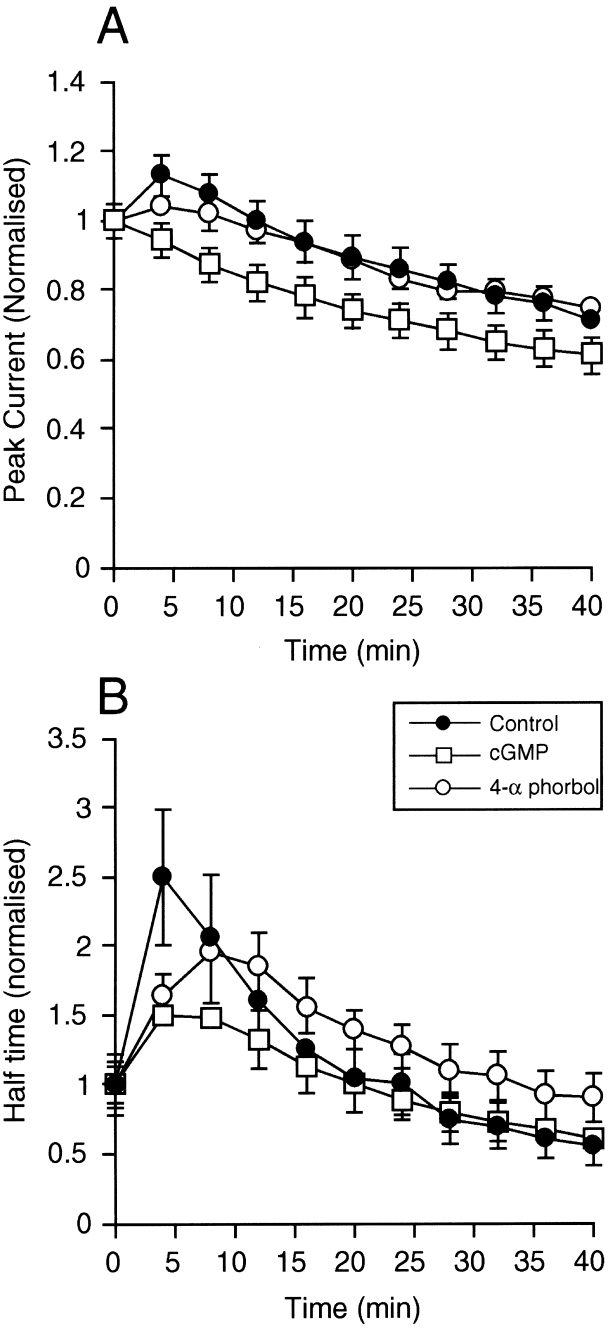

Fig. 5. Time course of $I_{5\text{-HT}}$ (A) and $t_{1/2}$ (B) of whole-cell currents recorded by patch-clamp from long form of murine 5-HT_{3A} receptors stably expressed in HEK 293 cells to repeated exposure to applications to 30 μM 5-HT in the absence (●, $n=5$) or in the presence of cGMP (□, $n=6$) or 4-α-phorbol (○, $n=6$). Data have been normalised to the first response ($t=0$). At no time point were any significant differences observed between the different conditions.

4.2. Effect of PMA

Inclusion of 100 nM PMA in the patch pipette also had varying effects on the long and short splice variants. At low concentrations of 5-HT PMA increased the peak amplitude of the response in the long but not the short

form of the receptor (Fig. 4). Similar results have been reported by Glitsch et al. [8] and Zhang et al. [25] who concluded that this is mediated by activation of PKC. Our combined data therefore suggest that PMA, in the short term and at low 5-HT concentrations, must either increase the number of functional receptors in the plasma membrane or increase the efficacy of the 5-HT_3 receptor. Because this effect is so rapid, it is unlikely to be the former, even though phosphorylation has previously been observed to stimulate assembly of the nACh receptor [9]. There is, however, previously reported evidence for an PMA-dependent increase in the mean single channel conductance, which would support the latter hypothesis: van Hooft and Vijverberg [21], using 5-HT_3 receptors expressed in N1E-115 cells, reported that PMA increased the probability of a high (27 pS) conductance level and decreased the probability of a much lower lever (6 pS), whereas the PKC inhibitor staurosporine had the opposite effect.

At high concentrations of 5-HT, PMA increased the rate of decay of the peak current (Fig. 3) in the long splice variant. Interestingly this increase would have the opposite effect to the enhancement discussed above, by reducing the total current through these receptors. The relatively long time-scale of this effect would support the hypothesis that PMA causes a reduction in the number of functional receptors, and might suggest that the long form of the 5-HT_3 receptor is phosphorylated by PKC and subsequently more rapidly internalised (i.e., receptor number is reduced) than the non-phosphorylated (short) form. There are two concensus sequences for PKC phosphorylation on the intracellular loop of the 5-HT_{3A} receptor subunit, although again these are present in both splice variants. However, as discussed above, the difference in the structure of the M3–M4 loop may modify the efficacy of phosphorylation, or alter the modulatory effects of one or more additional proteins which may be involved.

5. Summary

Thus, overall we have demonstrated that functional differences between long and short splice variants become apparent in the presence of cAMP and PMA. Both these agents have the potential to phosphorylate 5-HT_3 receptors, although it is something of a dilemma that the same consensus sequences for phosphorylation by PKA and PKC are present in both forms of the receptor. Nevertheless the additional six amino acids in the 'long' form may change the structure in such a way as to prevent or allow access of appropriate enzymes, resulting in differential phosphorylation. Alternatively additional proteins in the cell which are activated by the actions of cAMP or PMA could differentially modify the two forms of the receptor, again by recognising only one of the two structures exhibited by the splice variants. The differential effects modulated by these agents would results in significant functional differences in 5-HT_3 receptor function in vivo.

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