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# UNITARY PROPERTIES OF POTASSIUM CHANNELS ACTIVATED BY 5-HT IN ACUTELY ISOLATED RAT DORSAL RAPHE NEURONES

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SUMMARY

1. Single inwardly rectifying $K^+$ channel currents were recorded from acutely isolated adult serotonergic dorsal raphe (DR) neurones using the cell-attached and outside-out patch clamp configuration.

2. Four equally spaced conductance levels were observed in both outside-out and cell-attached patch recordings with conductance levels averaging 11, 21, 30 and 40 pS. Larger conductance openings (50–120 pS) were seen less frequently.

3. When using 136 $[K^+]_o$ the single channel *I–V* relation was linear in the range 0 mV to −100 mV in all cases.

4. Transitions between the various conductance levels were observed, as were apparent direct opening and closing to each individual conductance level. Furthermore openings of 11, 21 and 30 pS were observed in almost all the patches. These results suggest that the different-sized events result from substates of a single channel rather than several different channels with different conductances.

5. Unitary $K^+$ channel current probability of opening, recorded in cell-attached patch, was unchanged after 5-hydroxytryptamine (5-HT) was added to the bath outside the patch pipette which suggests that no easily diffusible second messenger was involved.

6. The single $K^+$ channel activity, however, was increased on average by 670% following the addition of 5-HT to the bath when recording channel activity in the outside-out configuration. Usually all $K^+$ channel subconductance levels increased in activity but the largest increases occurred in the events with 30 and 40 pS conductance.

7. These results suggest that 5-HT enhances the probability of opening of the resting $K^+$ channel activity, which can open to several levels of conductance, and that no new channel or freely diffusible second messenger is involved in the response.

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## INTRODUCTION

Ion channels selective for the passage of $K^+$ ions and which show varying degrees of inward rectification have been recognized since the work of Katz (1949). Most of the detailed work has been carried out on starfish egg cells, skeletal muscle, cultured myotubes, cardiac myocytes and cultured myotubes (Hagiwara & Takahashi, 1974; Leech & Stanfield, 1981; Sakmann & Trube, 1984*a*; Matsuda & Stanfield, 1989). Differences exist between the properties of channels found in these cells but certain basic similarities have emerged. The channels are activated by hyperpolarization from $E_K$ and their conductance is approximately proportional to $\sqrt{K_o^+}$. The channel open times are voltage dependent, shorter at negative potentials, and the channel is blocked by $Na^+$ ions; this process may contribute to the slow inactivation of the $K^+$ current seen after a hyperpolarizing voltage step. The inward rectifier channels in ventricular heart cells show conductance substates. In two of the studies investigators have found three equally spaced substrates which can open singly, or in a concerted fashion (Sakmann & Trube, 1984*a*; Matsuda, Matsuura & Noma, 1989).

Neurotransmitter receptor activation can modulate the activity of inwardly rectifying $K^+$ channels in some preparations, for instance the occupation of muscarinic receptors in heart activates an inwardly rectifying $K^+$ current and inward rectifiers in neurones also are activated by neurotransmitters (Williams, Colmers & Pan, 1988). Although studies of neurotransmitter activation of a $K^+$ channel in central neurones with properties suggestive of inward rectification have appeared, to date few have been carried out at the single channel level. Most of these studies used the cell-attached patch configuration and so were unable to wash neurotransmitter out of the pipette (Freedman & Weight, 1988; Miyake, Christie & North, 1989; VanDongen *et al.* 1988). In the present studies we have examined the serotonergic neurones of the dorsal raphe (DR) nucleus. It is known that 5-HT receptor activation opens $K^+$ channels in DR neurones in the brain slice preparation (Lakoski & Aghajanian, 1984). In our previous paper (Penington, Kelly & Fox, 1993) we characterized the $K^+$ current activated by 5-HT using whole-cell methods. We determined the conditions required to observe $K^+$ channel activation in acutely isolated adult neurones, and in this manuscript we have investigated the unitary properties of the channels activated by 5-HT.

## METHODS

Cell preparation was the same as that used in the accompanying paper (Penington *et al.* 1993).

*Single channel experiments*

Single channel experiments were performed using the cell-attached patch or outside-out mode of recording. The pipette contained (mM): KCl, 140; Hepes, 10; $CaCl_2$, 2; $MgCl_2$, 1; pH 7·4 with NaOH. The bath solution contained (mM): potassium aspartate, 140; K-EGTA, 10; $MgCl_2$, 1; Hepes, 10; pH 7·4 with KOH. This external solution has been shown to zero the resting potential of isolated neurones (Fox, Nowycky & Tsien, 1987). Therefore the potentials reported in this paper from cell-attached patches are absolute potentials. Liquid junction potentials were measured by first placing the pipette into the internal pipette solution (potassium aspartate) as a zero reference and then exchanging the bath solution. A liquid junction potential of 1 mV (relative to ground)

was measured between potassium aspartate and bath solutions containing KCl. The data in this paper have not been corrected for this small potential.

The outside-out patch experiments used the same pipette solution as used in the whole-cell experiments (Penington *et al.* 1993). The external solution contained (mM): KCl, 136; glucose, 10; Hepes, 20; $CaCl_2$, 2; TTX, 0·1 $\mu$M, pH 7·3 with NaOH. For the leak subtraction, sweeps where no channel openings were recorded were used. Leak subtraction consisted of averaging leak sweeps closest in time to the data sweeps. Current records were filtered by an 8-pole Bessel filter (Analog Devices) set to a corner frequency (−3 dB) of 2 kHz (when sampling at 10 kHz) and 1 kHz (when sampling at 5 kHz). The data were usually sampled at 100 and occasionally 200 $\mu$s per point.

An Axon Instruments Axopatch 1C was used as the amplifier throughout these experiments. Patch pipettes were pulled from 1·5 mm haematocrit glass (Scientific Products, McGaw Park IL, USA) and coated with Sylgard (Dow Corning Co., Midland, MI, USA). Polishing was done on a Narishige Microforge (model MF-83, Setagaya-KU, Tokyo). After polishing the patch pipette resistance for single channel experiments was between 3–5 MΩ, higher than we typically use for whole-cell recordings (1·5–2·5 MΩ). Electrophysiological recordings were done at room temperature (21–24 °C).

An IBM PC/AT clone computer running Axobasic (Axon Instruments, Foster City, CA, USA) was used to generate pulses, and to acquire and analyse data. Stimulus pulses were fed from a D/A converter in the computer to the patch clamp amplifier. Single channel patches were typically hyperpolarized once every second, unless indicated otherwise.

Single-channel patch clamp data were analysed with Pinhed (Axon Instruments), which provided single-channel amplitude histograms and probability of opening graphs. The analysis program modelled the single channel openings using a threshold method set at 50% of the single channel open current. Final plots of data were made using Coplot or Axobasic. Single channel seal resistance was always between 20 and 100 GΩ. All currents shown in this paper have been leak subtracted. In total, accumulated data from thirty-six patches are reported in this paper.

## RESULTS

### *Cell-attached patch recordings*

Single channel data were obtained from fourteen patches in the cell-attached patch configuration using equimolar $[K^+]$ inside and outside the electrode. Isotonic potassium aspartate was used externally to zero the membrane potential, as previously used in the study of $Ca^{2+}$ channels (Hess, Lansman & Tsien, 1984; Penington, Kelly & Fox, 1991). Membrane voltage was changed over the range −140 to +60 mV. Predominantly inward currents were observed below 0 mV; very few openings in the outward direction were seen (Fig. 1). Indeed, outward current openings were only observed positive to +40 mV, thus it was not easy to confirm that these openings were due to the same channel as the ones which were activated by hyperpolarization, below 0 mV.

As illustrated in Fig. 1 multiple conductance levels were always observed. In this case where the holding potential ($V_h$) was set at 0 mV (near the potassium reversal potential, $E_K$) and hyperpolarizing steps were applied, the cell exhibited $K^+$ channel currents characterized by conductance levels of 10, 20, 43, 60, 80 and 105 pS.

As can be seen in Fig. 1, the open times of the channels varied widely and were often interrupted by rapid closing perhaps between substates (see below). Fluctuations between fully closed and open states were also frequent. As more than one conductance level was always present, open time histograms for a single conductance level were not attempted.

In Fig. 2 the averaged unitary current amplitudes are plotted as a function of potential for several cell-attached patches. Different conductance levels are

immediately apparent. Sometimes only a few measurements could be made at given potentials due to the low occurrence of channel openings to a given substate. Thus in some cases only a limited number of patches could be used to calculate slope conductances. In all experiments a sufficiently large number of sweeps were always

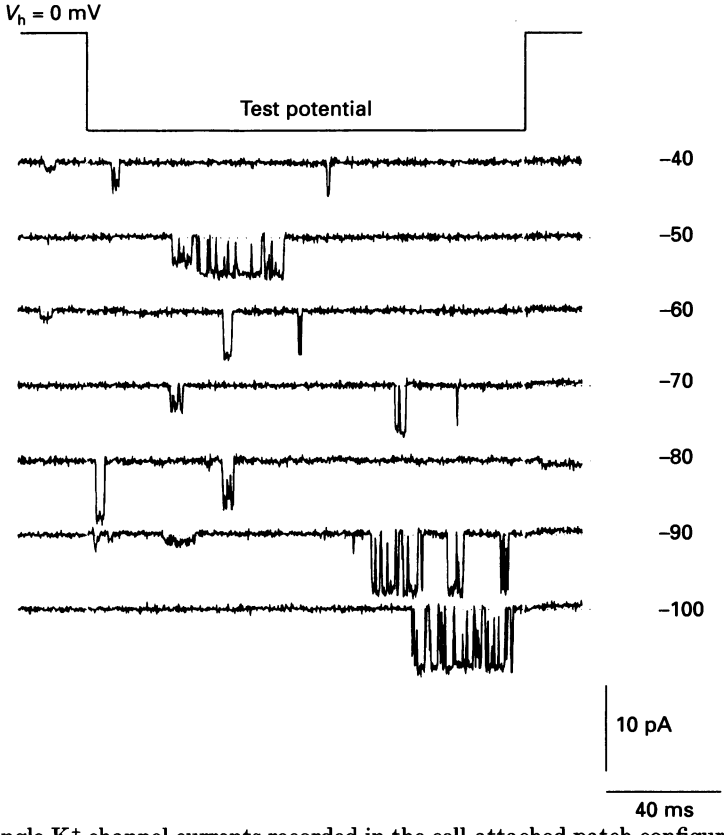


Fig. 1. Single $K^+$ channel currents recorded in the cell-attached patch configuration in an acutely isolated DR neurone. Unitary currents elicited by applying the hyperpolarizing potentials indicated from a holding potential ($V_h$) of 0 mV. The [$K^+$] inside the pipette and in the bath was 140 mM. Currents have been leak and capacitance compensated. The dotted lines indicate the zero current level. Although only four conductance levels are apparent in the sweeps plotted, this patch exhibited conductance levels of 10, 20, 43, 60, 80 and 105 pS.

taken at one potential to determine the channel amplitude. Assuming a reversal potential of 0 mV, the channel amplitude data was then converted into chord conductances.

In Table 1, chord and slope conductance levels have been averaged as they were essentially identical, but in the illustrations showing averaged *I–V* relations only the slope values are shown. For example, the events with a slope conductance value of 9 pS occurred in only two patches both of which showed enough openings at different potentials to obtain the average value. In contrast, chord conductances were obtained in every patch but, these averaged $11{\cdot}1 \pm 0{\cdot}6$ pS (mean $\pm$ S.E.M.) across the thirteen patches.

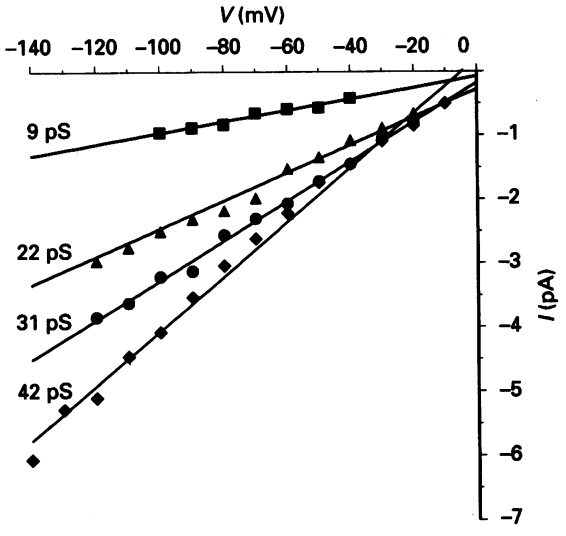


Fig. 2. Single channel current–voltage ($I$–$V$) plots constructed from several cell-attached patches. Slope conductances of 9 ($n = 2$), 22 ($n = 8$), 31 ($n = 4$) and 42 pS ($n = 4$), were observed. In these cases (except the 9 pS case where the S.E.M. was not calculated) the S.E.M. was smaller than the symbols. Very few openings were observed in the outward direction, suggesting inward rectification of the channel. The data are fitted well by the lines drawn (using a least-squares fitting routine), with the conductance values obtained from the slope of the lines. All the lines cross the voltage axis close to 0 mV, the theoretical value of $E_K$.

TABLE 1. Conductance levels observed in cell-attached patches

| Cell No. | $g$ (nS) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 13 | 25 | — | — | 48 | — | — | — | — | 124 |
| 2 | 11 | 22 | — | — | — | — | 70 | — | — | 117 |
| 3 | 12 | 20 | — | — | — | — | — | — | 98 | — |
| 4 | — | 25 | — | 40 | — | — | 70 | — | — | — |
| 5 | 11 | 20 | 30 | 41 | — | — | 75 | — | — | — |
| 6 | 15 | 22 | — | 37 | — | 61 | — | — | — | — |
| 7 | 12 | 19 | 28 | — | — | — | — | — | — | — |
| 8 | 10 | 20 | — | 43 | — | 60 | — | 80 | 105 | — |
| 9 | 12 | 21 | 29 | 42 | — | — | 69 | — | — | — |
| 10 | 8 | 22 | 30 | 43 | — | — | — | — | — | — |
| 11 | 8 | 23 | 28 34 | 39 | — | — | — | — | — | — |
| 12 | 9 | 20 | 28 | 40 44 | — | 65 | — | — | — | — |
| 13 | 14 | 25 | 31 34 | — | — | 62 | — | — | — | — |
| 14 | 10 | 18 | 34 | 45 | — | — | — | — | — | — |
| Mean ± S.E.M. | 11·1 ± 0·6 | 21·5 ± 0·6 | 29·1 ± 0·4 | 40·6 ± 0·7 | 48 | 62 | 71 ± 1·5 | 80 | 101·5 | 120 |

Most patches had four conductance levels. Seven patches had levels close to 10, 20, 30 or 40 pS. Three patches had five levels and three had three levels, while only one had six levels of conductance.

An average conductance level of approximately twice this level 21·5 ± 0·6 pS was measured in fourteen patches in which chord conductance measurements at one potential were also obtained. An average conductance of 29·1 ± 0·4 pS was also

calculated in eight patches and in nine patches enough data was obtained to calculate an average conductance of 40·6 ± 0·7 pS. Occasionally other levels were seen: 48, 61, 71, 80 101 and 120 pS. None the less, the four smaller more commonly observed conductance levels appeared to be equally spaced multiples of each other (Table 1).

The single channel *I–V* relation was linear between 0 and −100 mV but occasionally more negative to −100 mV the unitary channel current saturated so that the channel current amplitude did not change with further hyperpolarization. Similarly in the few experiments where channel openings were observed above 0 mV the outward currents were smaller, i.e. inward rectification was present. The lines drawn through the linear portion of the *I–V* relations cross the voltage axis close to 0 mV. On no occasion did the slope conductance increase with hyperpolarization as has been observed in single microelectrode recordings in DR slices (Williams *et al.* 1988) and single channel recordings in cultured noradrenergic locus coeruleus cells (Miyake *et al.* 1989). Single channels were observed in every patch indicating that the density of channels is quite high.

*$K^+$ channels in DR neurones exhibit multiple conductances and show evidence of substates*

In the next section we attempted to determine whether the multiple conductances result from discrete channels, or from single channels with several conductive pathways which could open separately or concertedly (Sakmann & Trube, 1984*a*; Matsuda *et al.* 1989). These conductive pathways probably result from one pore which has an adjustable size. If the separate levels represent the opening of separate channels the concerted opening of two or more channels should be relatively rare (Howe, Cull-Candy & Colquhoun, 1991) and only if substates are present will concerted openings occur relatively more frequently. When a channel exhibits substates it will be rare not to see several conductance levels in a given patch. If the different conductance levels are caused by several different channels in the same patch then in some patches certain conductance levels will be absent from the recording. The best evidence for substates in DR $K^+$ channels is the fact that every patch, whether recorded with cell-attached or outside-out methodology, showed a large number of events showing transitions between more than one level. Channels with substates show fluctuations between conductance levels, but the frequency of fluctuations need not be the same for every level (Cull-Candy & Usowicz, 1987).

Figure 3 shows single channel openings at three different potentials from three different cell-attached patches. In each, direct opening of the channel to different conductance levels can be seen. Figure 3*A* illustrates openings to three different current levels 2·8, 5·1 and 7·1 pA. In the initial part of the record transitions between the different current levels are annotated. At the point marked '1' the channel opens to the 5·1 pA level, closes fully '2' and then opens first to the 2·8 pA level and then directly to the 5·1 pA level '3', followed by a direct transition to the closed state '4'. The remainder of the record shows a variety of combinations of transitions to the three current levels.

Figure 3*B* illustrates openings selected from another cell-attached patch stepped to −130 mV which exhibits four levels of conductance. At the location marked '1' there is a direct opening to the 4·6 pA current level interrupted by a transition to a current level of 2·4 pA '2' for about 3 ms, the channel then opens directly to the

4·6 pA level '3' before closing completely. The notable point about this event is that the opening and closing of this two level event is a concerted or an apparent direct transition between full open, partially open, then fully open and finally a fully closed state. Concerted events should occur extremely rarely unless subconductance levels

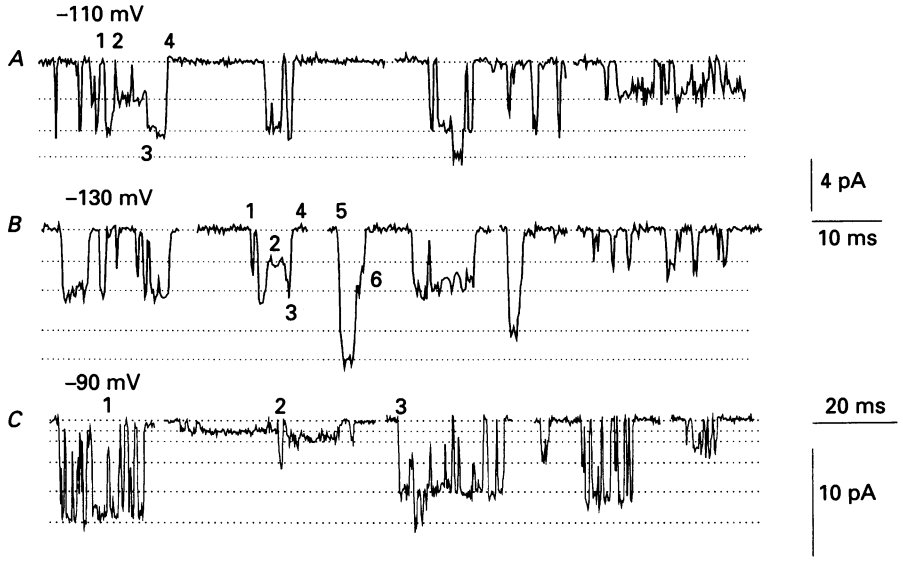


Fig. 3. Single channel recordings from cell-attached patches illustrating conductance substates in DR neurone $K^+$ channels. *A–C* data from three patches are shown. Each sweep represents several short duration portions of data, excerpted from longer data sweeps. The numbers, described in detail in the text, indicate either the concerted action of one or more channel or, more likely, the switching of a single channel between two conductance substates. Multiple current amplitudes were found in almost every patch. The patches were held at 0 mV with 80 ms steps to the potentials indicated were applied. Dotted lines are drawn at 0, 2·8, 5·1, and 7·1 pA in *A*, at 0, 2·4, 4·6, 7·6 and 9·7 pA in *B* and at 1, 1·9, 3·9, 6·6 and 9·4 pA in *C*.

are present. The opening to the largest conductance level in this patch (marked '5' occurs directly to the 9·7 pA level but during the closure of this channel there appears to be a pause at the 4·6 '6' and 2·4 pA current levels. The rest of this record illustrates the variety of conductance levels exhibited by this patch.

Panel *C* in Fig. 3 illustrates openings from a cell attached patch, held at −90 mV, from a different cell. Five levels of conductance were observed and current frequently switched between them. During the first group of openings (marked '1') switching occurred between the largest current level 9·4 pA and the lower conductance levels of the closed state. The second epoch of recording shows the current dwelling at the lowest level (1 pA) followed by a brief, large (not fully resolved) opening (marked '2'); this was followed by a brief, large (not fully resolved) opening (marked '2'); this was followed by a brief complete closure followed by a period at the second 1·9 pA level. At the location marked '3' the patch exhibited direct transitions between several current levels. The first transition is to the 6·6 pA level, followed by a closure to the 3·9 pA level and then an opening to the 9·4 pA level and a closure to the 6·6 pA

level. The opening then continues to exhibit occasional partial or complete closures. The comparative frequency of transitions between multiple levels observed in every patch coupled with apparent direct closure from the higher conductance state is indicative of multiple conductances of a single channel. The further evidence for subconductance states in DR neurones derives from the fact that the three smallest

TABLE 2. Conductance levels observed in outside-out patches and their sensitivity to 5-HT

| Cell No. | | | | *g* (nS) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1 | — | — | — | **38*** | — | — | — | — | — |
| 2 | 12 | 22 | 29 | **40** | — | — | — | — | 128 |
| 3 | 10 | 21 | 32 | **40** | — | — | — | — | — |
| 4 | 11 | 22 | 32 | **40** | — | — | — | — | — |
| 5 | 10 | 18 | **32** | — | — | — | — | — | — |
| 6 | **12** | **24** | **32** | **39** | — | **63** | **74** | — | **118** |
| 7 | 11 | 21 | **32** | — | — | — | — | — | **120** |
| 8 | 13 | 23 | 32 | 39 | — | — | **69** | — | — |
| 9 | **13** | **20** | **33** | **40** | — | — | **67** | — | — |
| 10 | 12 | 22 | 30 | — | **52** | — | — | — | — |
| 11 | 16 | 19 | **34** | — | — | — | — | — | — |
| 12 | 13 | 23 | 27 | 39 | **54** | — | — | — | — |
| 13 | 12 | 20 | **28** | — | — | 58 | — | — | — |
| 14 | 11 | 23 | 31 | 38 | — | — | — | 98 | — |
| 15 | 10 | 21 | 28 | 41 | 49 | — | — | — | — |
| 16 | 10 | 17 | 25 | 35 | 54 | — | — | — | 111 |
| 17 | 10 | 22 | 32 | — | — | — | — | — | — |
| 18 | 9 | 17 | — | — | 46 | — | 68 | — | 124 |
| 19 | 13 | 23 | 29 | — | 50 | — | — | — | — |
| 20 | 11 | 21 | **35** | **40** | — | — | — | — | **125** |
| 21 | 11 | **20** | **31** | — | — | **61** | — | — | — |
| 22 | 11 | 21 | **31** | 39 | — | **68** | — | — | — |
| Mean ± S.E.M. | 11·5 ± 0·3 | 20·9 ± 0·4 | 30·7 ± 0·5 | 39 ± 0·4 | 50·8 ± 0·6 | 60·5 | 69·5 | 98 | 121 ± 3 |

Conductance levels were measured in 22 patches. Like the cell-attached patch recordings most patches had levels of 10, 20, 30 or 40 pS. The conductance levels printed in **bold type** were observed to have a larger *NP* value in the presence of 5-HT. *The fact that only one level of conductance could be assigned with any certainty to this patch reflects the fast switching between levels, only one of which had an open time long enough for certain measurement.

conductances were seen in almost every outside-out patch (Table 1 and 2), and the majority of cell-attached patches.

*Single channel activity observed in the outside-out configuration*

When cell-free patches were recorded from in the outside-out configuration similar findings were noted to those obtained when using the cell-attached patch configuration. To obtain these data the same pipette solutions were used as in the accompanying whole-cell study and the external solution contained 136 mM $[K^+]_o$ (see Methods).

Figure 4*A* illustrates averaged single channel *I–V* relationships from outside-out patches. A patch with openings with an average slope conductance of 11 pS is illustrated. The average of both the chord and slope conductances was 11·5 ± 0·3 pS (mean ± S.E.M., $n = 21$; Table 2). Chord conductances that measured 20·9 ± 0·4 pS

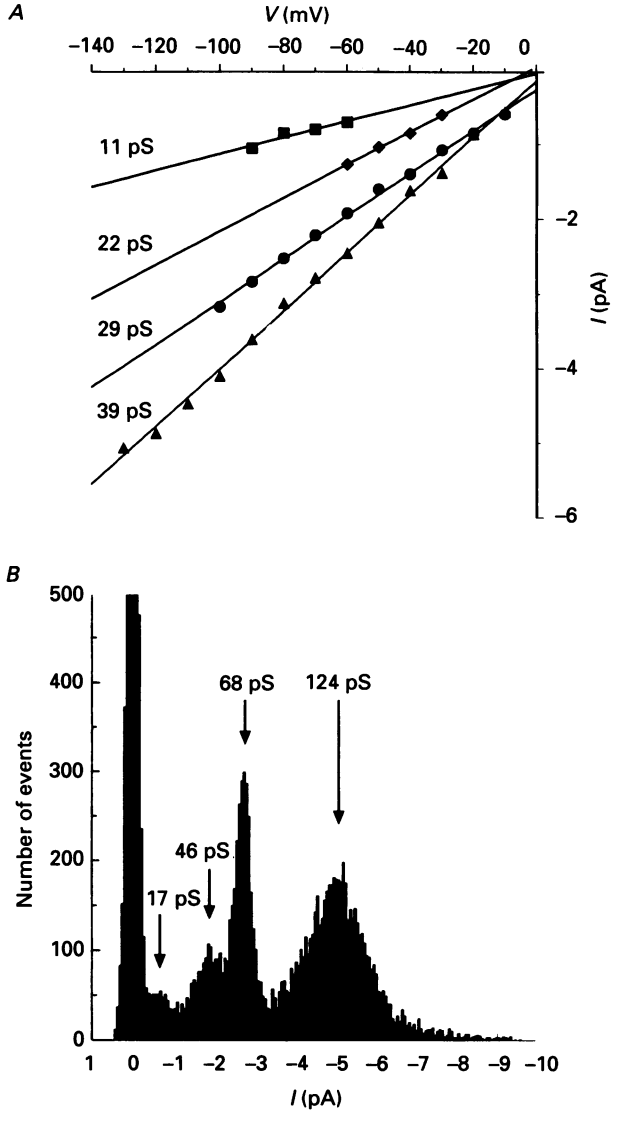


Fig. 4. Single channel *I–V* plots constructed from several outside-out patches. *A*, slope conductances of 11 ($n = 2$), 22 ($n = 1$), 30 ($n = 14$) and 39 pS ($n = 6$). In cases where a standard error was calculated it was usually smaller than the symbol size. Few openings were seen in the outward direction suggesting inward rectification. The data are fitted well by the lines drawn (using a least-squares fitting routine), with the annotated conductance values. All the lines cross the voltage axis close to $-3$ mV, the theoretical value of $E_K$. *B* shows an amplitude histogram from one outside-out patch (same patch as Fig. 5*C*). The patch was held at $-40$ mV and activity recorded. Four peaks of current can be distinguished corresponding to 17, 46, 68 and 124 pS conductance levels.

were also observed in every patch, i.e. values similar to those seen in the cell-attached patch data. A $30{\cdot}7 \pm 0{\cdot}5$ pS conductance level was observed in twenty patches. In agreement with the data gathered using cell-attached patch recording, thirteen of the

patches showed openings of conductance 39·0 ± 0·4 pS. Other levels were also observed: 50·8, 60·5, 69·5, 98 and 120·5 pS (Table 2). In nine out of twenty-two patches five levels of conductance were observed; seven patches had four levels and three patches had three levels. One patch each had six, seven, and one level of conductance.

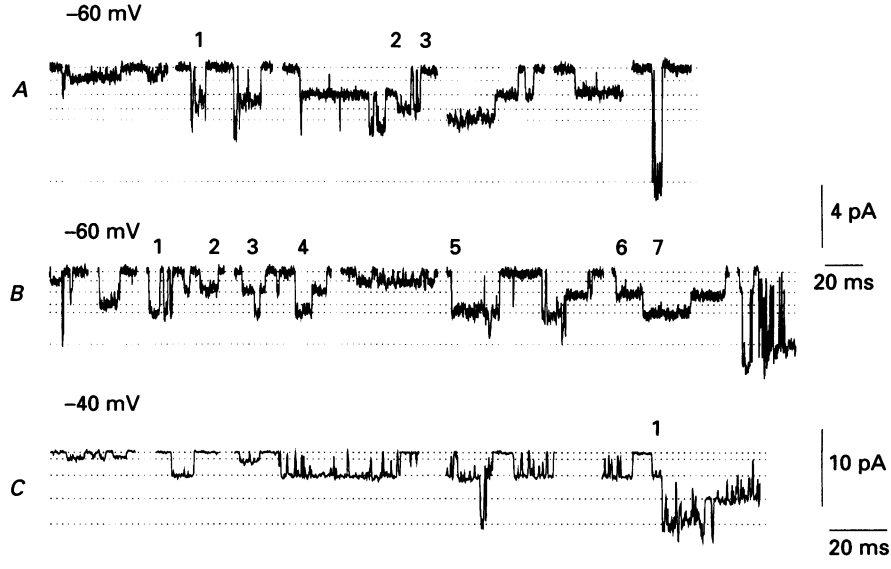


Fig. 5. Single channel recordings from outside-out patches illustrating conductance substates in DR neurone $K^+$ channels. Data from three patches are shown. Each sweep represents several short duration portions of data, excerpted from longer data sweeps. The numbers indicate transitions between current levels, and multiple current amplitudes were found in almost every patch; this is described in detail in the text. The patches were held at −60 mV or −40 mV and activity recorded. Dotted lines are drawn at the 0, 0·8, 1·7, 2·6, 3·3 and 7·2 pA current levels in *A*, at 0, 0·6, 1·3, 2·1, 2·5 and 4·6 pA in *B*, at 0, 0·9, 2·8, 5·9, and 9 pA in *C*. An amplitude histogram constructed from this patch is shown in Fig. 4*B*.

The *I–V* relations obtained using outside-out patches were almost identical to those measured using cell-attached patches; they were linear in most cells between −100 and 0 mV with very few openings in the outward direction. When these did occur they appeared to be partially blocked. Best-fit lines through the linear section of the *I–V* relations showed the single channel current reversed close to 0 mV.

Figure 4*B* shows a histogram of current amplitude obtained from one patch (No. 18, Table 2). At −40 mV four current peaks can be seen corresponding to 17, 46, 68 and 124 pS conductance levels. There was also an opening to 9 pS but this did not occur frequently at −40 mV. Figure 5*C* plots current records obtained from the same patch as Fig. 4*B*.

Outside-out patches also displayed multiple current levels and openings suggestive of the presence of substates rather than a concerted action between multiple channels in the patch. Figure 5 shows periods of single channel recording from three patches in three separate cells, two at −60 mV and one at −40 mV. The record in Fig. 5*A*

shows five levels of conductance which open and close in a fashion that is consistent with subconductance states. In Fig. 5*A* five different levels of current are observed. The opening marked '1' shows a transition between the third and second largest current levels. At the point marked '2' there is a transition in the opposite direction

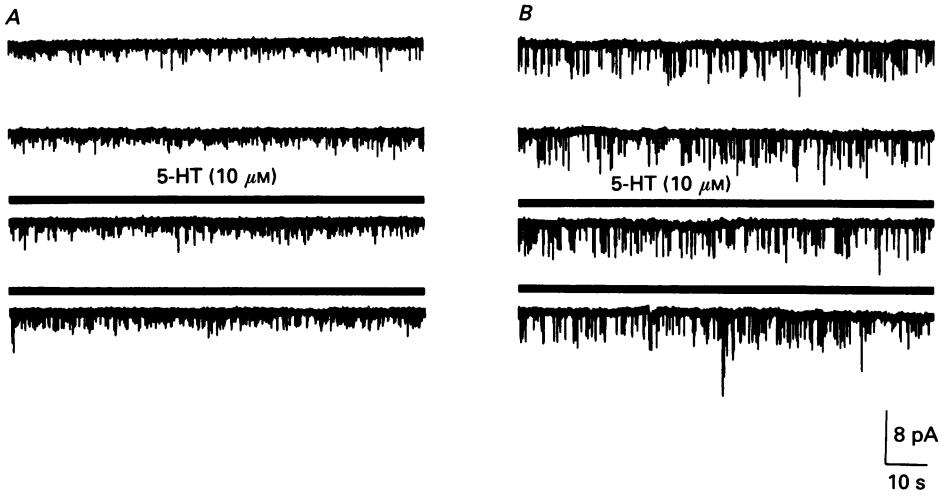


Fig. 6. 5-HT failed to increase $K^+$ channel activity in a cell-attached patch when it was added to the perfusion fluid outside the pipette. *A* plots excerpts from 400 s of $K^+$ channel unitary activity at $V_h = -60$ mV (no test depolarization). 100 ms of data were acquired every second for 400 s and then spliced together to form a continuous record. Thus 900 ms of every second was not recorded. The bar indicates the time during which 5-HT was added to the bath. In panel *A*, the channel activity (*NP*) was 0·029 in control conditions and 0·034 in the presence of 5-HT. Panel *B* was obtained by holding the patch at 0 mV and stepping the patch to −110 mV. The data has been leak and capacitance subtracted. The *NP* value was 0·030 in the control and 0·030 in the presence of 5-HT.

from the second to the third largest level and then a complete closure. The channel then directly opens and closes to the third largest current level twice (3) which is indicative of a discrete event and not the product of two events occurring simultaneously (Fox, 1987). Figure 5*B* also shows five levels of current. The openings marked '1' or a '2' are discrete transitions from the closed state to the 2·5 and 1·2 pA levels respectively. The openings marked '3' and '4' show transitions between these levels in both directions (from the 1·2 to the 2·5 pA level and from the 2·5 to the 1·2 pA level). The opening marked '5' shows a direct transition from the fully closed to the open and back to the fully closed state. The long opening marked '6' to the 1·2 pA level is interrupted by a transition to the 2·5 pA current level (7). The level marked '7' (2·5 pA) might be interpreted to result from two channels both of similar current amplitude (1·2 pA) opening simultaneously. This interpretation is quite unlikely, however, since the 2·5 pA current level not only occurs frequently but openings and closures occur directly to this level. Direct openings to and closures from the 2·5 pA level would be expected to occur only rarely if two separate channels were involved. These findings are consistent with a single channel with substates opening to different levels of conductance.

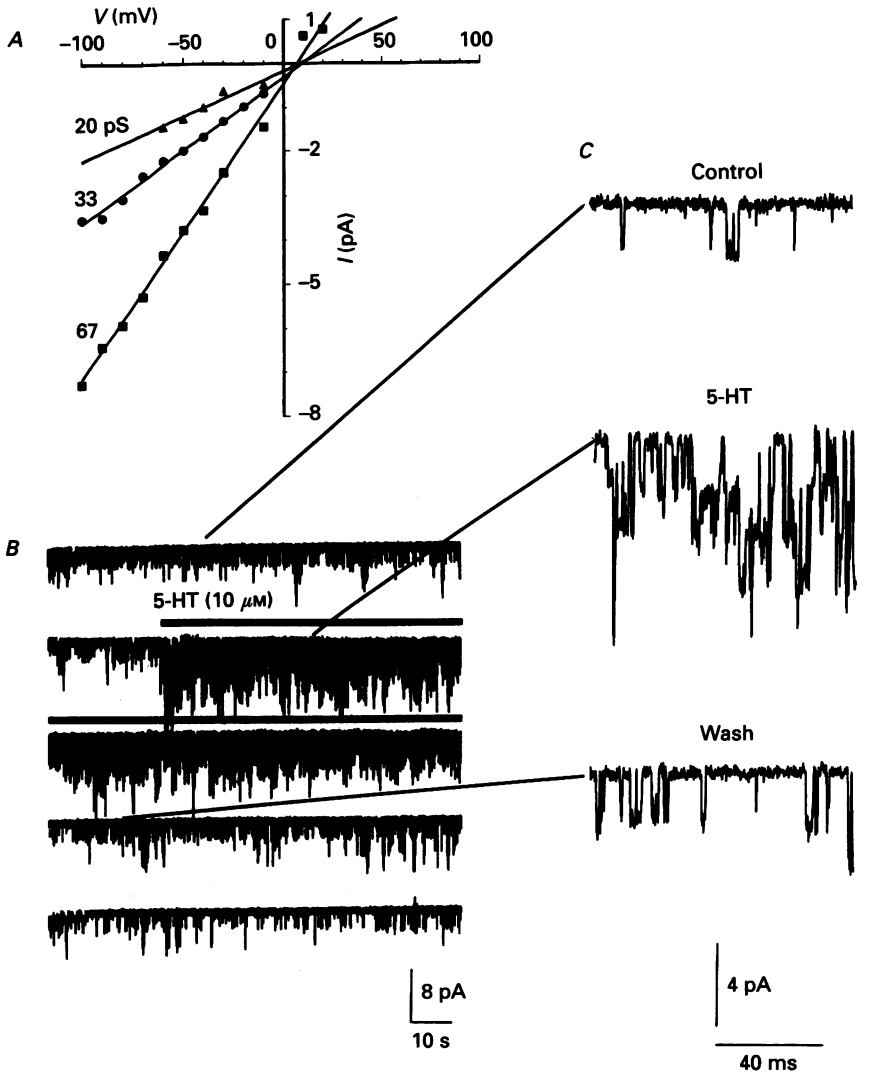


Fig. 7. 5-HT dramatically increased $K^+$ channel activity in an outside-out patch, when it was added to the bath. *A*, single channel *I–V* plots from the outside-out patch to which 5-HT was added. Five conductance levels were observed using both slope and chord conductance. *B*, unitary activity is plotted on a compressed time base, Activity was recorded by holding the patch at −60 mV. 100 ms of data was acquired every second and spliced together to form the continuous records shown (same parameters as in Fig. 6*A*). For the period indicated by the bars 5-HT (10 μM) was added to the bath; single channel activity increased dramatically. *C*, selected periods of the recorded activity are plotted on an expanded time base, obtained at the locations indicated by the lines.

*Single channel activity was not changed by 5-HT added to the bath in the cell-attached patch configuration*

In the accompanying paper (Penington *et al.* 1993) it was shown that 5-HT was capable of inducing an inwardly rectifying $K^+$ conductance in acutely isolated DR

neurones (Lakoski & Aghajanian, 1984; Williams *et al.* 1988). In the cell-attached patch configuration channels under the patch were not exposed to the 5-HT applied to the bath, thus any effect observed on the channels under these conditions should be due to an easily diffusible second messenger (Siegelbaum, Camardo & Kandel, 1982). 5-HT added to the bath during recordings from seven patches from seven cells in the cell-attached patch configuration failed to alter the channel activity (Fig. 6).

TABLE 3. 5-HT increased the $K^+$ channel opening (*NP*) in outside-out patches

| $NP_{Control}$ | $NP_{5\text{-}HT}$ | $NP_{Wash}$ | $NP_{5\text{-}HT}/NP_{Control}$ |
|---|---|---|---|
| 0·0860 | 0·143 | 0·0341 | 1·66 |
| 0·0197 | 0·0725 | — | 3·68 |
| 0·0381 | 0·0808 | 0·0382 | 2·12 |
| 0·0036 | 0·0238 | 0·0131 | 6·61 |
| 0·0526 | 0·6778 | 0·0696 | 12·88 |
| 0·1224 | 2·055 | 0·0179 | 16·78 |
| 0·0263 | 0·107 | 0·0012 | 4·06 |
| 0·182 | 0·947 | 0·148 | 5·20 |
| 0·0261 | 0·4264 | 0·0064 | 16·32 |
| 0·0129 | 0·0879 | 0·0108 | 6·81 |
| 1·151 | 2·017 | 0·637 | 1·75 |
| 0·0914 | 0·2447 | 0·0373 | 2·67 |
| Mean ± S.E.M. | — | — | 6·71 ± 1·67 |

In all cases the patch was held at −60 mV in 140 mM $[K^+]_o$. The same number of sweeps were used in all the different experimental conditions to calculate *NP*. The increase in activity over control is shown in the column on the right.

*Single channel activity is increased by 5-HT when recording in outside-out patches*

In the outside-out patch experiment illustrated in Fig. 7, 5-HT (10 μM, a maximal concentration) added to the bath could access the 5-HT receptor. The patch was held at −60 mV with no step in potential applied. Slope conductances measuring 20, 33 and 67 pS were obtained. Chord conductances for less frequent openings were also observed and measured 13 and 40 pS. These were distinct conductance levels rather than two openings of separate channels opening simultaneously. Below the *I–V* relation in Fig. 7*B* single channel activity is plotted on a compressed time scale. Each sweep corresponds to 100 s of activity. Some single channel activity was always present in the absence of any 5-HT in high $[K^+]_o$. When 5-HT (10 μM) was added to the bath the probability of channel opening ($P_{open}$) increased 5·2-fold. Representative sweeps are shown on an expanded time base in Fig. 7*C* in control, 5-HT and wash periods. 5-HT was added to twenty-two outside-out patches and fifteen of them responded with an increase in the probability of channel opening (Table 3). The response to 5-HT showed little or no desensitization, in agreement with whole-cell results (Penington *et al.* 1993).

Figure 8 displays an amplitude histogram from unitary currents shown in Fig. 7 in order to display the current level which is increased most by 5-HT. Three clear peaks can be seen during the control, 5-HT and wash periods of: 1·44, 2·22 and 4·33 pA corresponding to the three most frequent conductance levels. In the centre

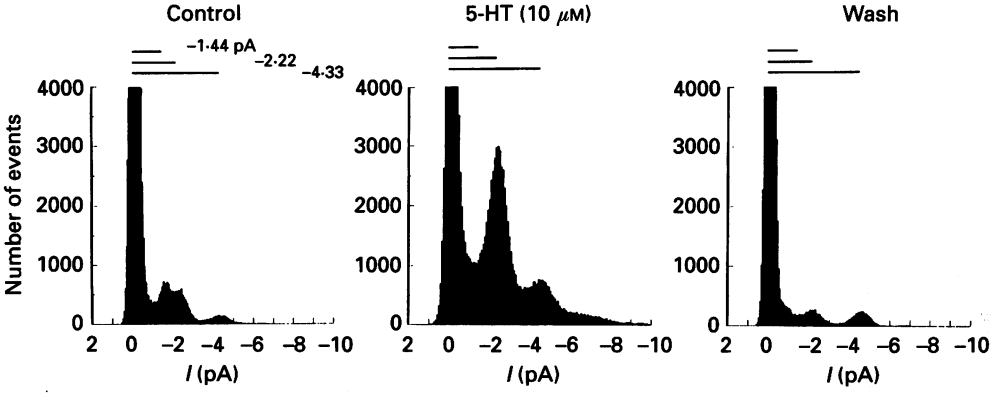


Fig. 8. $K^+$ channel amplitude histograms show the effect of 5-HT in more detail. Amplitude histograms were constructed for control (left), 5-HT (middle) and wash (right) conditions. The holding potential was −60 mV and 150 sweeps each of 100 ms length were used for each histogram. Three peaks were seen in all these conditions corresponding to 20, 33 and 67 pS. The 33 pS level increased most in the presence of 5-HT. This histogram is taken from the same patch shown in Fig. 7. The bars above the histogram show the position of the peaks at −1·44, −2·22 and −4·33 pA.

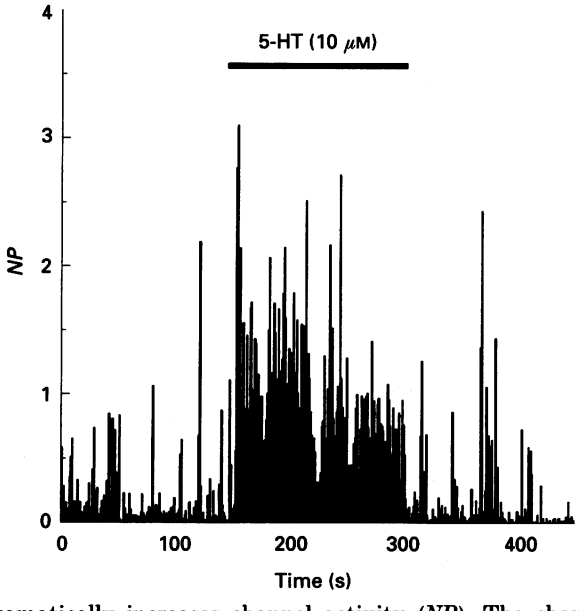


Fig. 9. 5-HT dramatically increases channel activity (*NP*). The channel activity was calculated for each recorded sweep. Each sweep is then plotted as a line in the histogram. The data is from the same patch illustrated in Figs 7 and 8. When 5-HT (10 μM) was added at sweep 150, *NP* increased 5-fold. When 5-HT was removed from the bath *NP* returned to control levels.

histogram although all three current peaks increase in the presence of 5-HT, the peak corresponding to the current level 2·22 pA (33 pS conductance) increased most. The peak at 6·5 pA presumably resulted from more frequent simultaneous openings of the

4·33 and 2·22 pA levels. A more general account of the conductance levels most likely to increase in 5-HT is given in Table 2. It can be seen that on some occasions almost every current level was seen to increase in 5-HT. In nine patches, however, a conductance level of about 30 pS was preferentially increased and in six patches one

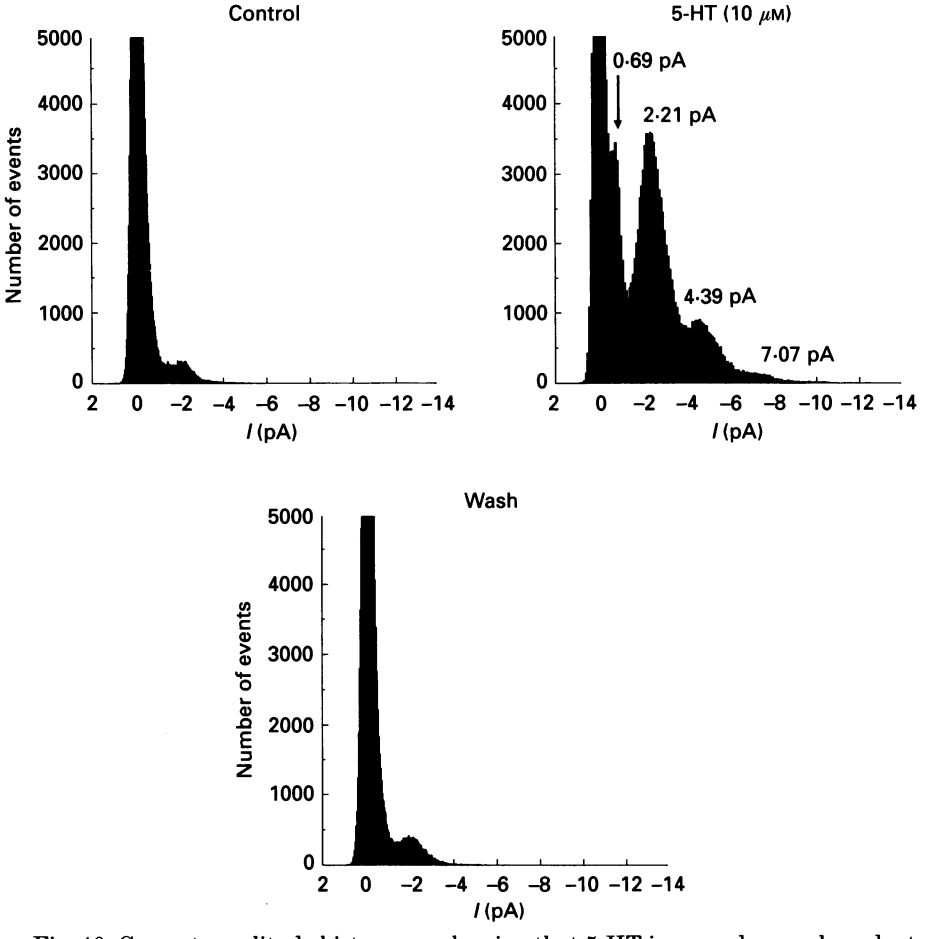


Fig. 10. Current amplitude histograms showing that 5-HT increased several conductance levels. Each histogram was constructed from 200 sweeps. The membrane potential was held at −60 mV. In 5-HT-free conditions (control) only one large peak was seen at 2·21 pA but in the presence of 5-HT four peaks were evident at 0·69, 2·21, 4·39 and 7·07 pA. All these levels of current were observed before 5-HT was added but much less frequently. Note that the 0·69 pA current level increased as much as the 2·21 pA level.

of about 40 pS increased most frequently. These findings are consistent with the suggestion that the conductance levels are substates of one another and that the 30 and perhaps 40 pS substate levels appear to be favoured in the presence of 5-HT. On the other hand if these current levels result from the simultaneous opening of completely separate channels, it would mean that 5-HT targets more than one type of $K^+$ channel.

A histogram of the channel activity (*NP*) is plotted against time for the data from the patch in the previous figure (Fig. 9). For each sweep activity is calculated and plotted as a bar in the histogram. Each sweep was made up of 100 ms of data, recovery every second. The channel activity during the control period was 0·182 and in the presence of 5-HT it increased to 0·947; during the wash it declined to 0·148. The channel activity during control, 5-HT and wash periods in twelve patches is documented in Table 3.

The finding that it is the 30 pS conductance events that were increased in the largest number of outside-out patches in the presence of 5-HT raises the possibility that a new 30 pS conductance is activated. Equally 5-HT could evoke a concerted multiple opening of a smaller subconductance whose sum is 30 pS. Figure 10 shows clearly that the levels 12, 39, 74 and 118 pS all increase (corresponding to 0·69, 2·21, 4·39 and 7·07 pA on the histogram). Inspection of the current records showed that less frequently observed levels of 24, 32 and 63 pS events also became more prevalent. These changes are difficult to observe in the amplitude histograms. This patch shows that the 11·5 pS openings (average value) increased as did the 39 pS openings. All of these levels open fully to the measured current level showing that they are conductance levels in their own right rather than simply simultaneous openings of separate channels. Indeed in most patches a 30 pS conductance level was nearly always present before 5-HT was added.

## DISCUSSION

The inwardly rectifying $K^+$ conductance of DR neurones has a number of characteristics in common with other preparations (skeletal muscle, cultured myotubes and starfish eggs); the inward rectification occurred near $E_K$ and the *I–V* relation was linear below $E_K$. Similar observations were made in whole-cell recordings reported in the accompanying paper (Penington *et al.*, 1993). However, in the present study the frequency of single channel openings was very low positive to $E_K$ in high $[K^+]_o$. Secondly, when openings did occur at this voltage they flickered to such an extent that the open channel current level was difficult to measure. It was also difficult to be certain if the openings above 0 mV were from the same channel(s) as seen in the hyperpolarizing direction. These observations explain why the *I–V* relations reported in this paper do not extend past 0 mV.

Another issue which is relevant to the identification of the channel which was activated by 5-HT is the degree of isolation of the $K^+$ channels characterized in this paper. As most data was gathered by holding the membrane potential constant at −60 mV in high $[K^+]_o$ several other types of $K^+$ channels were not activated; namely those which require a hyperpolarizing prepulse followed by depolarization to activate ($I_A$) or the delayed rectifier $K^+$ channels ($I_{DR}$) which only activate at more depolarized potentials. Both $I_A$ and $I_{DR}$ in whole-cell recordings were not altered by 5-HT in the earlier study (Penington *et al.* 1993). The possibility that some of the $K^+$ channel activity observed was calcium activated $K^+$ conductance ($I_{Ca}g_K$) appears unlikely because the channels were activated by hyperpolarizations which normally close $Ca^{2+}$ channels. Secondly, in the outside-out patches (which yielded data similar to cell-attached patches) 10 mM-EGTA in the patch pipette should buffer $[Ca^{2+}]_i$ and

thus inhibit $I_{Ca}g_K$. In whole-cell recording 10 mM EGTA in the pipette was sufficient to block $I_{Ca}g_K$ (Penington *et al.* 1992*b*).

The non-specific cation current $I_Q$ or $I_H$, activated by hyperpolarization, were not prominent features in whole-cell recordings from the same cells and so were unlikely to be activated by 5-HT in this study.

In skeletal muscle and cardiac cells the gating of inwardly rectifying $K^+$ channels is voltage dependent. Hyperpolarizing the cell produces a large instantaneous current which slowly decreases with time at negative potentials (Standen & Stanfield, 1979; Ohmori, 1980). The current decrease at negative potentials disappeared in high-$K^+$, $Na^+$-free solutions similar to those used in this study. In the present study the gating kinetics of the single channel currents in the absence of 5-HT were not obviously voltage dependent (saturation of the single channel current occurred on a few occasions at potentials below $-100$ mV; Penington *et al.* 1993).

Interestingly, inward rectification has been described as steep or mild. Mild inward rectification is thought to be due to a voltage-dependent block of the $K^+$ channels by $Mg^{2+}$ acting from the inside which reduces only outward movement of $K^+$ through the channel (Vandenburg, 1987; Matsuda, Saigusa & Irisawa, 1987) but steep inward rectification is due to a voltage-dependent gating process independent of a blockade by $Mg^{2+}$ (see Hille, 1991). When single channel currents were elicited in the outward direction they appeared to be partially blocked, possibly by $Mg^{2+}$ on the inside of the cell membrane but this occurred when using both methods of recording (physiological $[Mg^{2+}]$ in the cell-attached patch and 2 mM $Mg^{2+}$ with outside-out patches). Our whole-cell results indicate that in six cells when $Mg^{2+}$ was left out of the patch electrode there was no obvious effect on the inward rectification. This was done in order to ascertain if 5-HT could elicit a larger $K^+$ current in the outward direction in the absence of internal $Mg^{2+}$. ATP-sensitive $K^+$ channels in pancreatic $\beta$-cells (Ashcroft & Kakei, 1989) and cardiac $K^+$ channels also show voltage-dependent block by $Mg^{2+}$ with a dissociation constant, $K_D$, two orders of magnitude higher than in the inward rectifiers (Ashcroft & Kakei, 1989; Matsuda, 1991). In pancreatic $\beta$-cells, the presence of $Mg^{2+}$ reduces the effectiveness of channel block by ATP analogues (Ashcroft & Kakei, 1989) but has the opposite effect in cardiac myocytes (Findlay, 1988). DR neurone $K^+$ channels appear to resemble most the $K^+$ currents activated by muscarinic receptors in the heart (Horie & Irisawa, 1987) where the channels are gated by voltage. Even in the absence of internal $Mg^{2+}$, the 5-HT-activated channels pass much less current at more positive potentials.

Single channel cell-attached and outside-out patch data from DR neurones were comparable. This suggests that the pipette solution, containing as it did 2 mM MgATP was similar to the intracellular constituents of the cell.

When hyperpolarized, DR neurones usually show four equally spaced conductance levels, 11, 21, 30 and 40 pS. Larger conductance levels are seen less frequently. Sakmann & Trube (1984*a*, *b*) observed equally spaced conductance substates in cardiac ventricular myocytes of 7, 14, 21 and 28 pS and may indicate a conductance pathway about 30% smaller than that found in DR neurones. Matsuda *et al.* (1989) studied ventricular myocytes in guinea-pigs during partial channel blockade caused by $Cs^+$ or $Rb^+$. These workers found three equally spaced conductance pathways of 10, 20 and 30 pS. Interestingly neither group observed conductance levels larger than

those reported above. Serotonin increased the $P_{open}$ of $K^+$ channels in DR neurones with at least four different substates and predominantly activated the 30 and 40 pS levels. This raises a number of interesting possibilities; for example, a drug like the anxiolytic buspirone, thought to work as a partial agonist (Andrade & Nicoll, 1987*b*) on 5-$HT_{1A}$ receptors on DR neurones, may preferentially increase the activity of a different substate compared to 5-HT.

Evidence for a G-protein-mediated activation of $K^+$ channels in cultured hippocampal cells has been observed (VanDongen *et al.* 1988). The $G_o$ $\alpha$-subunit applied to inside-out patches activated four classes of $K^+$ channel activity. Under these conditions there were no freely diffusible second messengers present yet the channels were still capable of being activated. This is consistent with our results where no response was observed when 5-HT was added to the bath during cell-attached recording. Another similarity between hippocampal and DR neurones is that both exhibit subconductance states. In VanDongen *et al.* (1988) these were manifested as shoulders on amplitude–probability density plots which were not investigated further. It is relevant that several other similarities exist between the $K^+$ current activated in hippocampal cells and that current activated by 5-HT in DR neurones (Andrade & Nicoll, 1987*a*) specifically the inward rectification and the sensitivity to block by $Ba^{2+}$.

The fact that transitions occurred between conductance levels indicated the presence of substates. It is particularly noteworthy that the 30 and 40 pS conductance levels were favoured in 5-HT but *all* levels increased in activity. It is interesting to note that channels which have subconductance states usually exhibit the same sensitivity to blockers for each substate although transmitters can increase the activity of one over the others (Fox, 1987). If the different conductance levels that we observed represented separate channels, then 5-HT must be able to activate several different types of $K^+$ channel.

Recent whole-cell experiments have succeeded in demonstrating the expression of 5-$HT_{1A}$ receptors in cultured rat atrial myocytes using a recombinant vaccinia virus vector system. Receptors for acetylcholine are normally coupled to the activation of a $K^+$ current directly via a G-protein in these cells (Karschin *et al.* 1991). The internal perfusion of GTP-$\gamma$-S (a nucleotide analogue which irreversibly activates G-proteins) enhances the efficiency of transduction of the receptor-mediated effect to a subsequent pulse of agonist. This was interpreted by suggesting that the $K^+$ channel requires interaction with several G $\alpha$-subunits rather than just one, implying that the $K^+$ channel may have a number of independent subunits each capable of interacting with a G-protein. This raises some interesting possibilities for the interpretation of our results. Either the subunits can only open concertedly (to one conductance level) when a given number of G-protein $\alpha$-subunits have bound in which case the dose–response curve would show allosteric characteristics (very steep, with a high Hill coefficient, as observed by Kurachi, Ito & Sugimoto, 1990). Alternatively, the channel subunits may gate separately as suggested in the present study and each subunit could be gated by the binding of one, two, three or more G $\alpha$-subunits. Because it was found in this study that the large subconductance states can open directly or close to the zero current level directly, this model would predict the binding or unbinding of several G $\alpha$-subunits from the $K^+$ channel simultaneously

which seems to be kinetically unlikely. Perhaps the more likely scenario is that substates may be an intrinsic property of the $K^+$ channel. When the appropriate number of G $\alpha$-subunits have bound the channel can then open to any possible conductance level.

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